COMPLEMENTARITY BETWEEN MESSENGER RNA AND NUCLEAR RNA FROM HELA CELLS

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SUMMARY: Hybridization of labeled HeLa cell messenger RNA to HeLa cell nuclear RNA occurs as determined by ribonuclease resistance of annealed mixtures of the two RNA's. Hybrids were characterized by melting temperature and by centrifugation to equilibrium in Cs_2SO_4 gradients. No annealing to nucleolar RNA, f2 phage RNA or homopolymers, was detected. The apparent complementarity of nuclear RNA and messenger RNA probably reflects an aspect of messenger RNA synthesis.

INTRODUCTION

Messenger RNA (mRNA) in eucaryotic cells is defined as RNA which is associated with polyribosomes and which is released as ribonucleoprotein from polyribosomes upon their dissociation into subunits (1, 2). It is not presently understood how mRNA is synthesized. As a possible clue to the origin of mRNA we have investigated whether any base sequence complementarity exists between mRNA and nuclear RNA. To test such a possibility ^{3}H-labeled HeLa cell mRNA was prepared from polyribosomes and hybridized to unlabeled HeLa cell nuclear RNA. Ribonuclease resistance was used as a criterion of double-stranded RNA formed during hybridization.

METHODS

To prepare ^{3}H-uridine-labeled RNA, 4 x 10^7 HeLa cells were resuspended in 10 ml of Eagle's minimum essential medium (4) containing 0.04 μg/ml actinomycin D (5) and 1 μg/ml of ethidium bromide (6). After 30 minutes at 37°C, 10 ml of medium containing the same concentration of inhibitors plus 5 mC of ^{3}H-uridine (21 C/mM, New England Nuclear Corp.) was added. After 3 hours

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of labeling at 37°C, a cytoplasmic extract was made (7) and polyribosomes were separated by centrifugation in a gradient of 15-30% sucrose in reticulocyte standard buffer (RSB, 2). Fractions of the gradient containing polyribosomes were pooled and the polyribosomes concentrated by centrifugation (8). Messenger ribonucleoprotein was first released from polyribosomes and then separated from heterogeneous cytoplasmic RNA by sucrose-gradient centrifugation (1,2,8). The labeled messenger ribonucleoprotein sedimenting between 30 and 80S was identified by assaying aliquots of each fraction of the sucrose gradient for acid-insoluble radioactivity and concentrated by ethanol precipitation. The RNA was extracted with phenol (9). The final RNA preparation contained approximately 10^6 cpm with a specific activity for the labeled messenger RNA of approximately 3×10^5 cpm/μg RNA (10).

Unlabeled RNA was prepared from $1.2\text{-}1.6 \times 10^9$ HeLa cells by first separating the nuclei from the cytoplasm (7). Total cytoplasmic RNA was obtained by phenol extraction. Clean nuclei were obtained by a detergent wash (7) and then disrupted with high salt and digested with 40 μg/ml of electrophoretically purified pancreatic deoxyribonuclease (11). Nucleoli were pelleted from this mixture, washed, and phenol-extracted for nucleolar RNA. To obtain total nuclear RNA, the whole nuclear mixture was phenol extracted. The extracted nucleic acid was further digested for 30 minutes at 37°C with 200 μg/ml of deoxyribonuclease in a buffer containing 0.02 M Tris, pH 7.5; 0.005 M Mg acetate.

To remove any residual DNA, either the RNA was passed through a column of Sephadex G-100 and the UV-absorbing material in the void volume was ethanol precipitated or the RNA was precipitated two successive times in 2 M LiCl (12).

For hybridization, RNA mixtures were suspended in 2 x SSC

(standard saline citrate: 0.15M NaCl; 0.015 M Na - citrate) with 0.1% sodium dodecyl sulfate and 1% diethylpyrocarbonate, and placed in sealed micropipets which were submerged in a waterbath at 65° or 70°C. After annealing for the indicated times the micropipets were rapidly frozen on dry ice, the ends broken and the contents emptied into 2 ml of 2 x SSC. Ribonuclease resistance was determined as previously described (8,13).

RESULTS

The percentage of ribonuclease-resistant radioactivity in the mRNA varied in different preparations. With all preparations, annealing to nuclear RNA increased the percentage of ribonuclease resistance by three- to four-fold (Table 1). For instance, in experiment 1, the unannealed or self-annealed mRNA had about 3% ribonuclease-resistant RNA; annealing to nuclear RNA increased this value to 8-9%. Twenty-four hours of hybridization was sufficient to achieve close to maximal ribonuclease resistance.

To determine which fraction of nuclear RNA was hybridizing to mRNA, the nuclei were sub-fractionated into nucleoli and nucleoplasm. Only the RNA from the nucleoplasm protected the mRNA against ribonuclease (Table 1). This implicates the heterogeneous, nuclear RNA as the active species of RNA although further analysis must be done to show that some other nucleoplasmic species is not involved. Transfer RNA is not the active component because the active material precipitates in 2 M LiCl. Also, transfer and other small RNA's would not be found in the void volume after Sephadex G-100 filtration (14).

The specificity of the hybridization reaction with HeLa cell nuclear RNA was examined by annealing labeled mRNA to equal amounts of nuclear RNA, cytoplasmic RNA, f2 phage RNA, poly(A) and poly(I). Of these, only the nuclear and cytoplasmic RNA's

TABLE 1

Annealing of mRNA to Other RNA's*

Experiment number	Added unlabeled RNA	Time (hours)	Salt Concentration during ribonuclease digestion (xSSC)	% ribonuclease resistance
1	None	0	2.0	3.2
	None	27	2.0	2.3
	None	51	2.0	2.9
	Nuclear	1	2.0	3.0
	Nuclear	3	2.0	4.9
	Nuclear	27	2.0	7.8
	Nuclear	51	2.0	8.9
	Cytoplasmic	27	2.0	4.1
2	None	23	2.0	3.0
	Nucleolar	23	2.0	3.1
	Nucleoplasmic	23	2.0	12.2
3	None	21	2.0	1.9
	Nuclear	21	2.0	5.9
	Cytoplasmic	21	2.0	3.6
	f2 phage	21	2.0	1.2
	poly (A)	21	2.0	1.6
	poly (I)	21	2.0	2.0
4	Nuclear	29	2.0	9.0
	Nuclear	29	1.0	3.8
	Nuclear	29	0.5	1.8
	Nuclear	29	0.2	0.3

* For experiments 1 and 4 samples of either 3600 cpm of ^{3}H-mRNA alone or with 15 μg of nuclear RNA or 15 μg of cytoplasmic RNA were annealed at 65°C in a total volume of 25 μl. After the indicated times, half of the samples were digested with 15 μg/ml of ribonuclease A and 7.5 units/ml of ribonuclease T1. For experiment 2, samples of 2500 cpm of ^{3}H-mRNA were mixed with 10 μg of nucleolar RNA or with 25 μg of nucleoplasmic RNA in a total volume of 10 μl and annealed at 70°C for 23 hours, after which digestion was done with 50 μg/ml ribonuclease S and 25 units/ml of ribonuclease T1 in 0.1% sodium dodecyl sulfate. For experiment 3, 4800 cpm of ^{3}H-mRNA and 15 μg of each of the unlabeled RNA's were annealed and digested as described for experiment 1.

were active and the cytoplasmic RNA gave only half of the hybridization seen with the nuclear RNA (Table 1). The activity in cytoplasm has not been investigated further, partly because of the difficulty of ruling out contamination of the cytoplasmic RNA with nuclear RNA (1). The lack of activity in poly(A) and poly(I) indicates that neither poly(U) nor poly(C) sequences in mRNA are responsible for the hybridization. Because the mRNA is labeled with ^{3}H-uridine, poly(A) or poly(G) in the mRNA could not be the hybridizing species.

To investigate whether the ribonuclease-resistant material formed after hybridization behaved like double-stranded RNA, the product was subjected to the following tests: 1) susceptibility to ribonuclease at low salt concentrations; 2) thermal denaturation; 3) and centrifugation to equilibrium in cesium sulfate. Using the first of these criteria, a mixture of mRNA and nuclear RNA, which contained 9% ribonuclease-resistant RNA in 2 x SSC, gave only a residual of 0.3% ribonuclease resistance after digestion in 0.2 x SSC (Table 1, experiment 4).

For thermal denaturation, ^{3}H-mRNA containing 2500 cpm was annealed to 25 μg of nucleoplasmic RNA for 72 hours and the mixture was then heated in 2 x SSC. At temperatures between 60°-100°C samples were rapidly chilled and the percent of ribonuclease-resistant RNA was determined. The annealed molecules denatured over the broad temperature range of 80°-90°C with a T_m of 85°C (Figure 1). A residual 30% of the ribonuclease-resistant RNA at 60°C remained ribonuclease-resistant throughout the experiment. The lack of a sharp transition during thermal denaturation may be due to heterogeneity of size or base composition.

Equilibrium centrifugation in Cs_2SO_4 of the ribonuclease-resistant hybrid formed by mRNA and nuclear RNA showed that the average buoyant

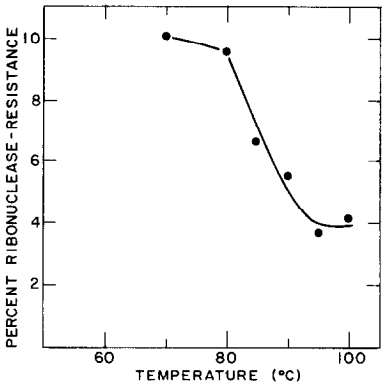

Fig. 1.

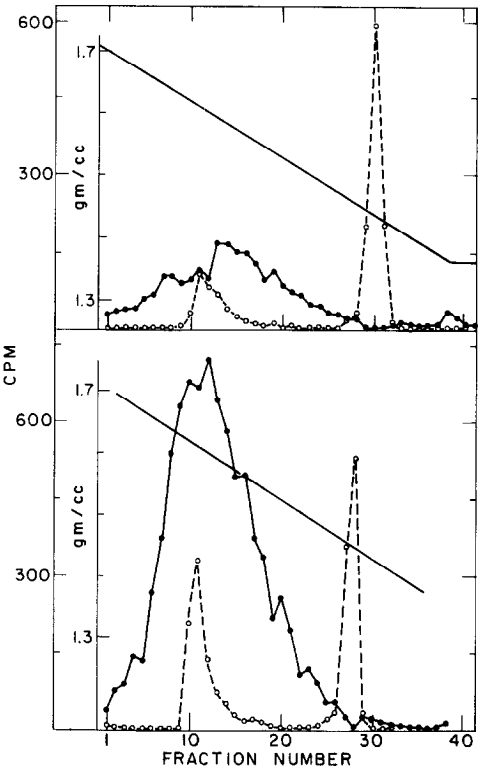

Fig. 2.

Fig. 1: Thermal denaturation of annealed product. Samples containing 2500 cpm of ^{3}H-mRNA and 25 μg of nucleoplasmic RNA were annealed in a volume of 10 μl at 70°C for 72 hours. Then the sealed micropipets were submerged in a beaker of water which was heated to 100°C within a 15-minute period. At the indicated temperatures a sample was removed, frozen and half of it digested with 50 μg/ml of ribonuclease A and 25 units/ml of ribonuclease T1 in 0.1% sodium dodecyl sulfate.

Fig. 2: Density of annealed product in Cs_2SO_4. Samples containing either 24,000 cpm of ^{3}H-mRNA alone (a) or with 600 μg of nuclear RNA (b) were annealed in a volume of 500 μl sealed in ampoules and incubated at 65°C for 21 hours, after which the samples were digested with 5 μg/ml ribonuclease A and 2.5 units/ml of ribonuclease T1 for 30 minutes at 22°C. Then the samples were mixed with 2% diethylpyrocarbonate for 1 hour before the addition of markers in the form of ^{14}C-labeled native bacteriophage DNA and poliovirus double-stranded RNA was added to the sample containing messenger RNA alone (a). Source of the markers and the methods for centrifugation in Cs_2SO_4 have been described (20).

density of the broad band of hybrid was about 1.60 gm/cc, identical to the value for added marker double-stranded poliovirus RNA (Figure 2B). This distinguishes the hybrids from DNA·RNA hybrids which have a density of about 1.51 gm/cc (15) and from double-stranded DNA (1.42 gm/cc; Figure 2). The ribonuclease-resistant portion of unannealed mRNA gave a broad distribution in Cs_2SO_4 (Figure 2A).

DISCUSSION

The data presented here show that there is base sequence homology between HeLa cell mRNA and nucleoplasmic RNA. When labeled mRNA taken from polyribosomes is annealed to nucleoplasmic RNA, RNA·RNA hybrids are formed which are ribonuclease-resistant, give a broad thermal melting profile with a T_m of 85°C and band in Cs_2SO_4 at the same density as poliovirus double-stranded RNA. Phage RNA and polypurines do not anneal to the mRNA but whole cytoplasmic RNA contains sequences complementary to mRNA. The only anomaly in these data is the low T_m relative, for instance, to ms-2 phage double-stranded RNA (16).

There are a number of possible explanations for these data including: 1) There could be sufficient fortuitous complementarity between nuclear RNA and mRNA to give rise to some ribonuclease resistance. 2) Regions of DNA could be transcribed in both directions and only one of these transcripts become mRNA. This situation would be analogous to that found in phage lambda (17). 3) There are double-stranded regions in the nuclear mRNA precursor (18,19). Cleavage of the precursor could occur between the regions of complementarity, yielding mRNA with one branch of the double-stranded RNA and nuclear RNA with the other branch. 4) By analogy with the mechanism of mRNA synthesis used by vesicular stomatitis virus (8), sequences of RNA

in nuclei could act as templates for the synthesis of some mRNA or of parts of mRNA. Such templates RNA would, in all liklihood, arise from transcription of DNA. The fourth explanation is the hypothesis which stimulated our experiments but the others, especially the third, seem equally likely.

REFERENCES

1. R.P. Perry and D. Kelley, J. Mol. Biol., 35, 37 (1968).
2. A.S. Huang and D. Baltimore, J. Mol. Biol., 47, 275 (1970).
3. J.E. Darnell, Bacteriol. Rev., 32, 262 (1968); J.E. Darnell, G.N. Pagoulatos, U. Lindberg and R. Balint, Cold Spring Harbor Symp. Quant. Biol., 35, 555 (1970); J.E. Darnell, L.Philipson, R. Wall and M. Adesnik, Science, 174, 507 (1971).
4. H. Eagle, Science, 130, 432 (1959).
5. R.M. Franklin and D. Baltimore, Cold Spring Harbor Symp. Quant. Biol., 27, 175 (1962); R.P. Perry, Proc. Natl. Acad. Sci. U.S., 48, 2179 (1962).
6. E. Zylber, C. Vesco and S. Penman, J. Mol. Biol., 44, 195 (1969).
7. S. Penman, J. Mol. Biol., 17, 117 (1966).
8. A. S. Huang, D. Baltimore and M. Stampfer, Virology, 42, 946 (1970).
9. M. Rosbash and S. Penman, J. Mol. Biol., 59, 227 (1971).
10. R. Soiero, M.H. Vaughan, J.R. Warner and J.E. Darnell, J. Cell Biol.,39, 112 (1968).
11. S. Penman, I. Smith and E. Holtzman, Science, 154, 786 (1966).
12. D. Baltimore, J. Mol. Biol., 18, 421 (1966).
13. D. Baltimore, A.S. Huang and M. Stampfer, Proc. Natl. Acad. Sci. U.S., 66, 572 (1970).
14. E.H. Davidson, M. Crippa, F.R. Kramer and A.E. Mirsky, Proc. Natl. Acad. Sci. U.S., 56, 856 (1966).
15. M. Chamberlin and P. Berg, J. Mol. Biol., 8, 297 (1964).
16. M.A. Billeter, C. Weissmann and R.C. Warner, J. Mol. Biol., 17 145 (1966).
17. K. Bøvre and W. Szybalski, Virology, 38, 614 (1969).
18. L.M. Montagnier, Compte Rend. Acad. Sci., Paris 267, 1417 (1968); L. Harel and L. Montagnier, Nature New Biology, 229, 106 (1971); C. Colby and P.H. Duesberg, Nature 222, 940 (1969); P.C. Kimball and P.H. Duesberg, J. Virol., 7, 697 (1971); R. Stern and R.M. Friedman, Biochem., 10, 3635 (1971).
19. W. Jelinek and J.E. Darnell, Proc. Natl. Acad. Sci. U.S., in the press.
20. K.F. Manly, D.F. Smoler, E. Bromfeld and D. Baltimore, J. Virol., 7, 766 (1971).

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