STUDIES ON HELA CELL NUCLEAR DNA-LIKE RNA BY RNA-DNA HYBRIDIZATION*

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A class of rapidly labeled nuclear RNA distinct from ribosomal precursor RNA has recently been characterized in various types of animal cells.[1-6] This RNA has a base composition resembling DNA (substituting uracil for thymine) and is therefore clearly distinguishable from nucleolar ribosomal RNA precursor molecules. On the basis of sedimentation behavior after various treatments[3, 6] and contour lengths in electron micrographs,[2] it has been suggested that this "DNA-like" RNA represents molecules of various lengths, including some polyribonucleotide chains as large as 10^7 molecular weight. We have used the abbreviation HS-nRNA (heterogeneously sedimenting nuclear RNA) to refer to this class of RNA obtained from HeLa cells.[5, 6] The cytoplasmic mRNA of HeLa cells comprises molecules which sediment much more slowly than the HS-nRNA,[7, 8] although the base composition of both is "DNA-like." It is therefore possible that cytoplasmic mRNA is derived from HS-nRNA by a specific cleavage mechanism(s). Such a mechanism, by which long polynucleotides are converted to specific shorter molecules, has been described in HeLa cells: 28*S* and 16*S* ribosomal RNA derive from a 45*S* ribosomal precursor molecule in the nucleolus of the cell.[6, 9, 10]

Work on nucleated duck erythroblasts (cells which primarily synthesize hemoglobin) suggests that the majority of HS-nRNA molecules in that cell never leave the nucleus but are apparently constantly synthesized and degraded.[2, 3] On the other hand, consideration of the kinetics of pulse-chase experiments have led Brown and Gurdon to suggest that in developing frog embryos at least some of the shorter DNA-like RNA may arise from cleavage of longer molecules. In HeLa cells, the rate of incorporation of H^3-uridine into HS-nRNA was compared to what would be expected if the HS-nRNA served as a precursor to cytoplasmic mRNA.[5] Those kinetic experiments gave no clear indication of whether any of the HS-nRNA might be converted to cytoplasmic mRNA.

To explore further the relationship of these two classes of "DNA-like" RNA molecules, we turned to the technique of RNA-DNA hybridization.[11] It was anticipated that experiments utilizing competition hybridization would be especially useful. For example, if HS-nRNA or RNA derived from it never entered the cytoplasm, hybrid formation by radioactive HS-nRNA should not be affected by cytoplasmic mRNA. It was found, however, that while unlabeled HeLa cell cytoplasmic RNA would interfere with hybridization of labeled RNA molecules, no true competition for DNA sites by unlabeled RNA molecules could be demonstrated. Therefore, the extent to which nucleotide sequences in these two classes of DNA-like RNA might be related could not be determined from such experiments.

Materials and Methods.—Labeling procedures and preparation of nucleic acids: Hela cells were grown, labeled, and fractionated into cytoplasmic and nuclear fractions as previously described.[5, 7, 10] The total nuclear RNA was extracted by a hot phenol-sodium dodecyl sulfate (phenol-SDS) procedure and subjected to zonal sedimentation.[6, 12] The RNA sedimenting faster than the 45*S* ribosomal precursor peak (approximately 50–100*S*) was collected by ethanol pre-

cipitation and dissolved in 2 × SSC (SSC, standard saline citrate = 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7.4). Radioactively labeled cytoplasmic messenger RNA was prepared from cells exposed to H^3-uridine for 30 min either by phenol extraction of the total cytoplasm followed by differential precipitation of soluble RNA and higher-molecular-weight RNA by an isopropanol precipitation step,[14] or by phenol extraction of cytoplasmic particulates obtained after sedimentation at 100,000 *g* for 90 min.[15] RNA isolated by either procedure had the sedimentation characteristics of messenger RNA. RNA was prepared from whole L cells which had been labeled in a manner similar to the procedure for HeLa cells and a fraction was obtained which sedimented more rapidly than 45*S*.

B. subtilis phage 2C messenger RNA was prepared from infected cells by the method of Pène.[16] All samples containing labeled RNA were prepared for scintillation counting as described previously.[17]

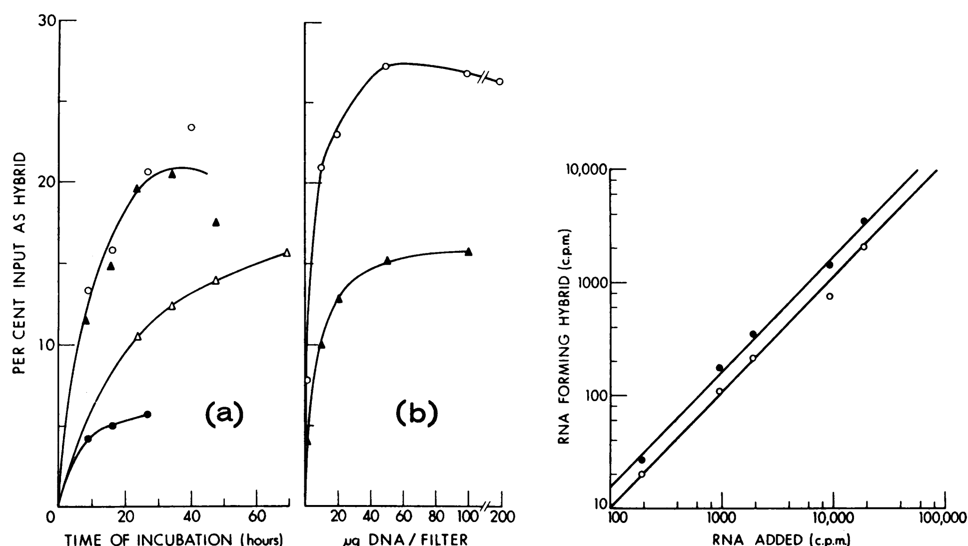
Unlabeled HeLa cell RNA was prepared in batches by the hot phenol technique from the cytoplasmic extract of 2 × 10^9 cells. In some RNA preparations, contaminating polysaccharide (presumably glycogen) was removed as described by Ralph and Bellamy.[18] L cell cytoplasmic RNA was a gift of R. Bellamy.

DNA was prepared from HeLa and L cells by the method of Marmur[13] as was bacterial DNA.

RNA-DNA hybridization technique: DNA was immobilized on nitrocellulose filters as described by Gillespie and Spiegelman.[11] Retention of C^{14}-thymidine-labeled DNA to Millipore filters was tested and found to be over 90% with less than 1% loss in 4 days of incubation at 60° in 2 × SSC. RNA-DNA hybridization was performed by placing a filter in 1 ml of a solution of 2 × SSC containing radioactive RNA. (All RNA samples were boiled 10 min in 2 × SSC and quickly chilled prior to addition to the hybridization reaction mixture.) Incubations were carried out in plastic scintillation vials at 60° for 24–48 hr. These conditions were found to be optimal for hybrid formation. To measure *total hybrid* all liquid was aspirated from the vial, and the filter was washed twice with 20 ml of 2 × SSC at 60° for 30 min. The filter was removed and dried; radioactive RNA remaining bound to the filter was assayed by scintillation counting. *RNase-resistant hybrid* was determined as follows. The liquid in the vials was removed and 5 ml of 2 × SSC containing 2γ/ml of crystalline pancreatic RNase (previously heated at 80°) and 5 units/ml of T-1 RNase were added. The filters were incubated for 30 min at 37° and washed with 20 ml of 2 × SSC for 30 min at 37°. Residual bound radioactivity was then determined. In a few experiments, the nuclease treatment of RNA-DNA hybrids consisted of 10 γ/ml pancreatic ribonuclease at 22° for 30 min in 2 × SSC.

Results.—Properties of the hybrid: The first experiments investigated the effects of the time of incubation, and of the amount and kind of DNA on the filters, on the binding of radioactive HS-nRNA. Figure 1 shows that approximately 15–20 per cent of the added HeLa RNA was bound to HeLa DNA as total hybrid, while about 3–5 per cent of the input was bound as RNase-resistant hybrid. In both cases, a period of at least 24 hours was required for maximum hybrid formation. Table 1 shows that the hybrid was specific, as shown by low binding of HS-nRNA to *E. coli* DNA filters. Figure 1 shows that with 10γ of DNA per filter at least 75 per cent as much hybrid formed as with 50γ, and that no increase in the amount of hybrid was observed above 50γ of DNA per filter. These results suggest that whereas at 10γ, DNA sites are actually present in excess, the *rate* of formation of hybrid was dependent upon the amount of DNA on the filter.

Additional evidence that in such experiments the amount of DNA was not limiting is seen in Figure 2. Here, the amount of input RNA was varied over a 100-fold range while the amount of DNA was kept constant at 10γ filter. The percentage of input RNA bound to the DNA either as total or RNase-resistant hybrid was independent of the amount of RNA added. In an attempt to determine what fraction of the total DNA was in fact able to react with the nuclear RNA, i.e., "saturation"-type experiments, very small amounts of DNA 0.25–2.5γ (2.5 × 10^4 to



(*Left*) FIG. 1.—Variables in formation of RNA-DNA hybrids. (*a*) Radioactive HS-nRNA RNA from HeLa cells was incubated under hybridization conditions (see *Methods*) with filters bearing either 10γ or 50γ of HeLa cell DNA for the indicated times. Either total hybrid or RNase-resistant hybrid was then determined by digestion with both pancreatic and T-1 RNases. All points in this and subsequent figures and all entries in tables represent averages of duplicate or triplicate values. ○, ▲ 50γ filters, total hybrid (2 separate expts.); △ 10γ filters, total hybrid, and ● 50γ filters, RNase-resistant hybrid. (*b*) A constant input of radioactive HeLa cell RNA was hybridized for 48 hr with filters bearing varying amounts of HeLa cell DNA. Total hybrid was measured. ○ HS-nRNA; ▲ cytoplasmic mRNA.

(*Right*) FIG. 2.—Effect of varying input of HS-nRNA on extent of hybridization. Radioactive HS-nRNA was prepared from HeLa cells and amounts ranging from the equivalent of 2×10^3 to 2×10^6 cells was hybridized with 10γ of DNA. Total hybrid (●) as well as hybrid resistant to pancreatic RNase was determined (○).

2×10^5 cell equivalents of DNA) and relatively large amounts of HS-nRNA, the equivalent to the HS-nRNA of about 10^7 cells, were incubated in liquid together. Incubation in liquid was necessary at low DNA concentrations because of the slower rate of hybrid formation when DNA is immobilized to filters.[11] In such experiments the fraction of input RNA which formed a hybrid decreased slightly at high RNA/DNA ratios, although no definite plateau was found. This failure to obtain saturation of the DNA suggests that the HS-nRNA molecules are not transcribed from a restricted portion of the cellular DNA.

Consideration was given next to the degree of specificity of the interaction between DNA and RNA, the stability of the binding, and the relative resistance to RNase conferred upon the RNA molecule when associated with DNA as a presumably hydrogen-bonded, helical structure.

TABLE 1

SPECIFICITY OF RNA-DNA HYBRIDIZATION WITH HELA AND L CELL RNA

Source of RNA	Input (cpm)	Source of DNA, (cpm in hybrid) HeLa	L cell	*E. coli*	No DNA
HeLa cytoplasmic mRNA	38,200	1183 (440)	349 (52)	164 (54)	119 (44)
HeLa HS-nRNA	9,100	1592 (389)	157 (40)	56 (30)	50 (30)
L cell HS-nRNA	6,900	238 (49)	590 (125)	49 (38)	31 (31)

Details of hybridization and hybrid estimation in *Methods*. "Total hybrid" given outside parentheses; cpm in parentheses represent RNase-resistant hybrid after digestion with both pancreatic RNase and T-1 RNase.

Binding of RNA to filters without DNA or nonspecific binding to totally unrelated segments of DNA was measured as that fraction of RNA which bound to filters bearing no DNA or to *E. coli DNA* filters (Table 1). A more stringent test of specificity was the ability of the RNA to discriminate between two more closely related DNA's, that from HeLa cells, a human strain, and L cells, a mouse strain (Table 1). The formation of both total hybrid and RNase-resistant hybrid exhibited a high degree of specificity, the RNase-resistant hybrid showing a somewhat greater discrimination between homologous and heterologous DNA's. As tested by RNase resistance, HeLa cell RNA hybridized with HeLa cell DNA at least 20 times better than with L cell DNA and conversely, L cell RNA bound to its homologous DNA at least 5 times better than to HeLa cell DNA.

Animal cell RNA-DNA hybrids were compared to a phage messenger RNA-DNA hybrid with respect to resistance to RNase (Table 2). The *B. subtilis* phage 2C was used. As might be expected from the relative complexities of the genomes, a larger fraction of input phage RNA formed a hybrid, and a larger proportion of that was resistant to RNase.

Competition studies using large amounts of unlabeled animal cell RNA as competitor: The foregoing experiments indicate that, as with phage and bacterial messenger RNA, DNA-like animal cell RNA forms specific RNA-DNA hybrids. With phage messenger RNA, another type of experiment termed "competition hybridization" has been used to determine the similarity or difference between mRNA species derived from the same DNA genome at different times after infection.[20, 21] In such experiments, labeled RNA is incubated with homologous DNA in the presence of relatively large amounts of unlabeled RNA. A decrease in the amount of hybrid formed as a function of the amount of unlabeled RNA added has been taken to indicate competition between labeled and unlabeled RNA molecules for the same sites on the DNA. The failure of heterologous RNA has been interpreted as indicating specificity in such a competition study.

Recently, Kasai and Bautz[22] have introduced an important variation in competition hybridization experiments. Since the RNA-DNA hybrid is stable once formed, these authors reason that a more sensitive procedure for testing whether a competing unlabeled species of RNA is truly occupying (saturating) available DNA sites is to preincubate the DNA filter with the competing unlabeled RNA. After the filter is washed to remove nonhybridized RNA molecules, it can be tested to determine whether the DNA can still form hybrid molecules with labeled RNA molecules. They found that phage DNA could in fact be saturated by unlabeled phage mRNA molecules, and that subsequent hybrid formation with labeled molecules of the same type was depressed.

TABLE 2

STABILITY OF "TOTAL HYBRID" TO NUCLEASE DIGESTION

RNA	Total input (cpm)	Cpm Bound after Nuclease Digestion for (min) 0	30	60
HeLa HS-nRNA	3,050	610 (20%)	199 (6.7%)	150 (5%)
HeLa cyto mRNA	4,250	295 (7%)	107 (2.5%)	112 (2.5%)
Phage 2C mRNA	3,370	1120 (32%)	890 (25%)	870 (25%)

RNA-DNA hybridization was carried out for 24 hr with homologous DNA as described in *Methods;* RNA bound was measured before, and 30 and 60 min after treatment with pancreatic ribonuclease plus T-1 ribonuclease (see *Methods*).

These considerations have been ignored by workers attempting to do competition hybridization with animal cell nucleic acids.[23–25]

We have therefore approached competition experiments with the point of view that interference with hybrid formation must first be proved to be due to occupation of DNA sites by unlabeled molecules. For the "competition" experiment shown in Figure 3*a*, labeled HS-nRNA and labeled cytoplasmic messenger RNA were prepared separately and incubated with HeLa DNA in the presence of increasing amounts of unlabeled cytoplasmic RNA. With both species of labeled RNA the amount of hybrid formed was greatly depressed by the unlabeled cytoplasmic RNA. However, several lines of evidence suggest that this interference of hybrid formation *was not due to true occupation of DNA sites by unlabeled RNA molecules.*

As shown in Figure 3*a*, unlabeled HeLa cytoplasmic RNA can inhibit the formation of a hybrid between L cell DNA and labeled L cell RNA by at least 55 per cent. It is most unlikely that human HeLa RNA molecules are "identical" (identical in base sequence over the length of a cistron) with the mouse L cell RNA molecules they have displaced. When the amino acid sequences of proteins with similar functions from different species are compared, it is found that an average of at least 1 amino acid in 10 is different.[26] Our data provide additional evidence that the RNA molecules from the two cell lines are not identical: L cell RNA formed a hybrid with HeLa DNA only 10 per cent as well as with homologous DNA, and HeLa RNA formed a hybrid with L cell DNA only a few per cent as well as with HeLa DNA (Table 1). These considerations point to major differences in sequences in the HeLa and L cell nuclear RNA.

The nonspecific aspects of "competition" experiments performed by simultaneous addition of labeled and unlabeled RNA species are further evidenced in Figures 3*a* and *b*. Unlabeled L cell RNA inhibited the formation of hybrid by HeLa RNA with its DNA and both unlabeled HeLa and L cell RNA inhibited the formation of hybrid by *B. subtilis* phage 2C messenger RNA with its DNA. It was extremely unlikely that a component other than RNA was responsible for the observed inhibition, the most direct evidence being that when HeLa cell RNA was treated with RNase, it no longer inhibited hybrid formation in any system. In addition, a number of treatments aimed at further purification of HeLa cell cytoplasmic RNA did not affect its capacity to inhibit hybridization. Among these

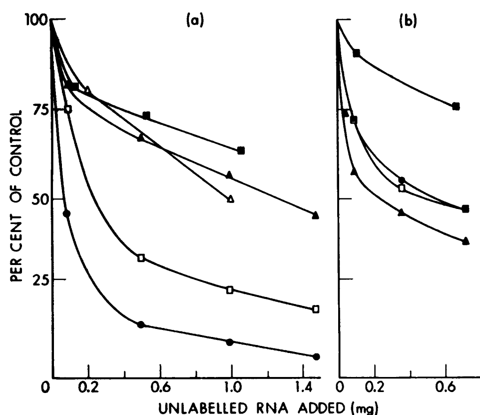


FIG. 3.—Interruption of hybrid formation by unlabeled HeLa and L cell RNA. Various radioactive RNA preparations were hybridized with filters containing homologous DNA in the presence of indicated amounts of unlabeled RNA. Nuclease-resistant hybrid (pancreatic plus T-1 RNase) was determined at the end of 24 hr. Similar results were obtained in experiments in which total hybrid was determined. (*a*) Unlabeled RNA = HeLa cell cytoplasmic RNA; ● labeled HeLa cell cytoplasmic nRNA; □ labeled HeLa cell HS-nRNA; △ labeled L cell HS-nRNA, 30–50*S*; ▲ labeled L cell HS-nRNA > 50*S*; ■ labeled phage 2C mRNA. (*b*) Unlabeled RNA = L cell cytoplasmic RNA. Symbols as in (*a*).

treatments were (1) additional 60° extraction with SDS and phenol, (2) removal of contaminating high-molecular-weight polysaccharide by the method of Ralph and Bellamy,[18] (3) precipitation with isopropanol under conditions which precipitated high-molecular-weight RNA only,[14] (4) isolation of molecules sedimenting between 6*S* and 30*S* by sucrose gradient sedimentation, and (5) DNase digestion. The observed depression of hybrid formation would appear to be a property of animal cell RNA, since neither *E. coli* nor yeast RNA had any effect on hybrid formation by labeled HeLa cell RNA.

Caution in interpreting interference with hybrid formation by animal cell RNA as valid "competition" is also strongly suggested by the results shown in Table 3. HeLa DNA-containing filters were exposed for 24 hours to amounts of unlabeled HeLa cytoplasmic RNA which, if added simultaneously with labeled RNA molecules, would have caused a 50–70 per cent reduction hybrid formation. The filters were then washed thoroughly and exposed to radioactively labeled HS-nRNA or cytoplasmic messenger RNA from HeLa cells. No inhibition of hybrid formation could be detected with either type of labeled molecule, i.e., there had been no "presaturation" of DNA sites by the unlabeled molecules. (It is relevant to note that, just as for bacteriophage mRNA-DNA hybrids, HeLa cell hybrids, once formed, were stable at 60° for at least 48 hr.) If the unlabeled RNA was not removed at the end of the first 24 hours, and labeled cytoplasmic RNA was then added, the inhibition observed was about the same as if unlabeled and labeled RNA were added simultaneously.

Discussion.—Hybrid molecules between rapidly labeled "DNA-like" RNA from cultured animal cells or tissues and homologous and heterologous DNA's have been repeatedly demonstrated by various techniques.[9, 19, 23, 27] In the present experiments, the specificity of the reaction involving formation of hybrid has been clearly

TABLE 3

Stability of RNA-DNA Hybrids at 60° and Failure of Unlabeled RNA to Presaturate DNA

Expt.	Time (hr) 0–24	24	24–48	Hybrid (cpm)
1	Hot			320
	2 × SSC		Hot	318
	Hot	Wash	Cold	274
	Cold	Wash	Hot	372
	Cold + Hot			145
	Cold		Hot	139
2	2 × SSC		Hot	75
	Cold		Hot	25
	Cold	Wash	Hot	66
	2 × SSC		Cold + Hot	29
3	Hot	Wash	2 × SSC	474
	Hot			450
	Hot	Wash	Cold	441
4	Hot			760
	Hot	Wash	2 × SSC	827
	Cold	Wash	Hot	774
	Cold		Hot	361
	Hot		Cold	771

In expts. 1 and 2, input radioactive RNA was cytoplasmic mRNA and RNase-resistant hybrid was determined. In expts. 3 and 4, input radioactive RNA was HeLa cell HS-nRNA and total hybrid was determined. In all experiments, 50γ of DNA per filter was used.

Explanation of symbols: hot = incubation of filter with radioactive RNA at 60° (see *Methods*), cold = incubation of filter with 0.6 or 0.7 mg unlabeled cytoplasmic RNA at 60° (see *Methods*); wash = procedure outlined in *Methods* for washing filters at 60° with buffer; 2 × SSC = incubation offilter in 1 ml of 2 × SSC at 60°.

demonstrated. HS-nRNA and cytoplasmic mRNA from HeLa cells hybridized with HeLa DNA better than with L cell DNA; the reciprocal experiment also demonstrated considerable specificity.

Previous experiments have shown that there is at least five times more radioactive HS-nRNA than cytoplasmic mRNA in briefly labeled cells;[6] in addition, the HS-nRNA forms hybrids with greater efficiency than cytoplasmic mRNA (e.g., see Fig. 1 and Table 2). The reports describing the hybridization of total rapidly labeled RNA from animal cells have clearly measured primarily HS-nRNA hybrids, not mRNA. Therefore, all rapidly labeled and readily hybridizable RNA cannot be equated with mRNA.

The technique of competition hybridization was used in an attempt to detect similarities between HS-nRNA and cytoplasmic mRNA. If found, this would imply that long polynucleotides containing cytoplasmic mRNA are direct products of gene transcription, and that these are subsequently cleaved into smaller molecules to be used as cytoplasmic mRNA. Unlabeled HeLa cell cytoplasmic RNA was found to depress hybrid formation by labeled HS-nRNA, but it also interrupted L cell RNA-DNA hybridization as well as phage 2C mRNA-DNA hybridization. Moreover, when HeLa cell DNA was exposed to unlabeled HeLa cell RNA (an amount sufficient to depress labeled HS-nRNA hybridization by 60% if added simultaneously), there was no stable preoccupation of DNA sites; labeled HS-nRNA, added after the cold RNA, hybridized to the same extent as if both were added simultaneously. This contrasts sharply with results obtained with phage systems, where stable "preoccupation" of DNA with unlabeled RNA has been unequivocally demonstrated.[22] We therefore conclude that it is not possible to quantitate the degree to which two classes of DNA-like RNA molecules from animal cells are related employing competition hybridization involving simultaneous addition of unlabeled RNA.

Unlike animal RNA, unlabeled RNA from yeast or bacterial cells did not depress hybrid formation nonspecifically. Two possibilities may be suggested to explain the nonspecific interference of animal cell RNA with hybrid formation. There may be RNA-RNA interaction which prevents true hybrid formation. The suggestion is not that helical duplex RNA-RNA formation occurs (this is ruled out by the accessibility of all nonhybridized RNA to RNase), but rather of a more general reaction, e.g., between ribosomal RNA and the DNA-like RNA. A second possibility is that the genomes of animal cells possess many genes accumulated during evolution which are similar but not identical. For example, the various globin chains (α, β, γ, fetal, etc.) and myoglobins are similar in many stretches of their primary amino acid sequences.[26] Messenger RNA molecules from such a gene might form an imperfect hybrid with a closely related gene, and thereby prevent the exact RNA gene copy from forming a true hybrid. Upon removal of excess RNA, the imperfect hybrid could dissociate and expose available DNA sites. Such a reaction would not occur with bacterial mRNA and DNA, because they presumably lack these similar but nonidentical repetitious sequences. Whether these speculations are valid is less important than the clear implication of the present experiments that competition hybridization with animal cell materials must be rigorously controlled to ensure that a presumed competing reaction is due to true occupation of DNA sites. It should be pointed out that these studies do not rule out the possibility of adjusting experimental conditions so that in competition ex-

periments true occupation of DNA sites can be demonstrated, but the present results do suggest that this has not yet been done with any species of animal cell DNA-like RNA.

Summary.—RNA-DNA hybridization has been used to investigate the relationship between cytoplasmic messenger RNA and nuclear DNA-like RNA of HeLa cells. It has been found that each of these kinds of RNA was able to form highly specific hybrids with DNA. By contrast, when a large amount of unlabeled cytoplasmic RNA was added simultaneously with radioactive RNA in "competition" studies, inhibition of hybrid formation occurred in a nonspecific manner. It is concluded that such "competition" experiments with animal cells cannot serve to prove the existence of identical molecules within two classes of RNA.

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[1] Yoshikawa-Fukada, M., T. Fukada, and Y. Kawade, *Biochim. Biophys. Acta*, **103**, 383 (1965); Ellem, K. A. O., and J. W. Sheridan, *Biochem. Biophys. Res. Commun.*, **16**, 505 (1964); Kubinski, H., and G. Koch, *Biochem. Biophys. Res. Commun.*, **22**, 376 (1966); Murumatsu, M., J. L. Hodnett, and H. Busch, *J. Biol. Chem.*, **241**, 1544 (1966).

[2] Scherrer, K., L. Marcaud, F. Zajdela, I. M. London, and F. Gros, these PROCEEDINGS, **56**, 1571 (1966).

[3] Attardi, G., H. Parnas, M.-I. H. Hwang, and B. Attardi, *J. Mol. Biol.*, **20**, 145 (1966); Houssais, J. F., and G. Attardi, these PROCEEDINGS, **56**, 616 (1966).

[4] Brown, D. D., and J. B. Gurdon, *J. Mol. Biol.*, **19**, 399 (1966).

[5] Soeiro, R., H. C. Birnboim, and J. E. Darnell, *J. Mol. Biol.*, **19**, 362 (1966).

[6] Warner, J. R., R. Soeiro, H. C. Birnboim, M. Girard, and J. E. Darnell, *J. Mol. Biol.*, **19**, 349 (1966).

[7] Penman, S., K. Scherrer, Y. Becker, and J. E. Darnell, these PROCEEDINGS, **49**, 654 (1963).

[8] Girard, M., H. Latham, S. Penman, and J. E. Darnell, *J. Mol. Biol.*, **11**, 187 (1965).

[9] Scherrer, K., H. Latham, and J. E. Darnell, these PROCEEDINGS, **49**, 240 (1963).

[10] Penman, S., *J. Mol. Biol.*, **17**, 117 (1966).

[11] Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).

[12] Eagle, H., *Science*, **130**, 432 (1959).

[13] Marmur, J., *J. Mol. Biol.*, **3**, 208 (1961).

[14] Zubay, G., *J. Mol. Biol.*, **4**, 347 (1962).

[15] Latham, H., and J. E. Darnell, *J. Mol. Biol.*, **14**, 13 (1965).

[16] Pène, J. J., and J. Marmur, *J. Virology*, **1**, 86 (1967); Pène, J. J., in preparation.

[17] Penman, S., Y. Becker, and J. E. Darnell, *J. Mol. Biol.*, **8**, 541–545 (1964).

[18] Ralph, R. K., and A. R. Bellamy, *Biochim. Biophys. Acta*, **87**, 9 (1964).

[19] Perry, R. P., P. R. Srinivasan, and D. E. Kelley, *Science*, **145**, 504 (1964).

[20] Hall, B. D., A. P. Nygaard, and M. H. Green, *J. Mol. Biol.*, **9**, 143 (1964).

[21] Green, M. H., these PROCEEDINGS, **52**, 1388 (1964).

[22] Kasai, T., and E. Bautz, *Proc. Rutgers Symp., September* 1966, in press.

[23] Whiteley, A. H., B. J. McCarthy, and H. R. Whiteley, these PROCEEDINGS, **55**, 519 (1966).

[24] Glišin, V. R., M. V. Glišin, and P. Doty, these PROCEEDINGS, **56**, 285 (1966).

[25] Church, R. B., and B. J. McCarthy, *J. Mol. Biol.*, **23**, 459, 477 (1967).

[26] Eck, R. V., and M. D. Dayhoff, *Atlas of Protein Sequence and Structure* (Silver Spring, Maryland: National Biomedical Research Foundation, 1966).

[27] Hoyer, B. H., B. J. McCarthy, and E. T. Bolton, *Science*, **140**, 1408 (1963).