*RECOGNITION OF RIBOSOMAL RNA SITES IN DNA, II. THE HELA CELL SYSTEM**

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As reported in a preceding paper,[1] the analysis of the sedimentation behavior and base composition of the RNA recovered from hybrids between *E. coli* 16*S* and 23*S* RNA and homologous DNA, after RNase digestion of non-base-paired segments, has given results which suggest a regular and complete hydrogen bonding of the hybridized RNA with specific sites in DNA. A similar analysis has now been applied to the investigation of ribosomal RNA sites in HeLa and other human DNA's and has made it possible to resolve the ambiguities in specificity pattern and base composition presented by the raw hybridization data, and to identify among the RNA-DNA complexes a fraction which appears to involve specific sites in DNA.

Materials and Methods.—Methods of growth in suspension of HeLa cells have been described previously.[2] Ribosomal RNA labeled to a high specific activity with P^{32} was prepared by growing the cells for 48 hr in a modified Eagle's medium[3] with 4.5×10^{-5} *M* phosphate and 5% dialyzed horse serum, in the presence of 20–25 μC/ml P^{32} carrier-free orthophosphate. In order to reduce the specific activity of the unstable RNA fraction,[4] the cells were subjected to a chase for 16–20 hr in the presence of 10^{-2} *M* unlabeled phosphate.

Ribosomal RNA was extracted from the ribosomal fraction[2] with redistilled, water-saturated phenol containing 0.1% hydroxyquinoline,[5] in the presence of 0.5% sodium dodecylsulfate, 5 mg/ml naphthalene disulfonate,[5] and 1.3 mg/ml bentonite.[6] Following 3 ethanol precipitations, the final product was dissolved in acetate buffer 0.01 *M*, pH 5.0, NaCl 0.1 *M*, and resolved into the 28*S* and 18*S* components by two cycles of sucrose gradient (linear, 5–20%) centrifugation. Several P^{32}-labeled preparations made in this way had at the time of isolation specific activities varying between 1.0 and 1.5×10^6 dpm/μg RNA. While the 28*S* preparations were always found to be free of any detectable P^{32} contaminant (less than 10^{-6}), determined as RNase-resistant counts excluded by Sephadex G-100 or alkali stable counts, the 18*S* preparations usually showed between 0.1 and 1% RNase-resistant radioactivity associated with macromolecular material, which could be reduced to less than 10^{-4} by two or more passages of the 18*S* preparation through nitrocellulose membranes.[7]

Reference is made to the preceding paper[1] for the procedures followed for DNA preparation and denaturation, RNA-DNA hybridization (incubation here was carried out at 70°C for $2^1/_2$–8 hr), isolation of hybrids, and analysis of sedimentation properties and base composition of hybridized RNA after RNase digestion.

Results.—Preliminary experiments carried out by incubating a constant amount of HeLa DNA with increasing amounts of 28*S* RNA indicated that the DNA could apparently be saturated at levels around 2×10^{-4}. Cross-hybridization tests (Table 1), however, yielded an ambiguous specificity pattern, suggesting the presence in the RNA-DNA complexes being examined of a large proportion of hybrids resistant to RNase involving irrelevant DNA segments. The existence of this background was further hinted at by the results of base composition analysis. When the hybrid was separated by Sephadex chromatography in low ionic strength medium (TNC) with or without a second RNase digestion after the gel filtration, the base composition of the hybridized RNA was very different from that of the original RNA (Table 2); in particular, the high purine to pyrimidine ratio suggested an extensive attack by

TABLE 1

COMPARATIVE OVER-ALL LEVEL OF HYBRIDIZATION OF HELA 28S RNA WITH VARIOUS DNA'S

DNA source	μg 28S RNA complexed/μg DNA × 10^4
HeLa	1.9
HeLa (native)	0.1
HeLa (without annealing)	1.0
Adult human liver	1.7
Fetal human liver	2.1
Adult human brain	1.9
Fetal human brain	2.1
Adult human kidney	2.5
Calf thymus	2.1
Duck blood	2.0
Pea embyro	2.6
Neurospora crassa	5.8
E. coli	0.63
T4	0.07

Each annealing mixture contained 25–50 μg/ml DNA and 1/10 this concentration of 28S P^{32} RNA. Incubation was at 70°C for 21/2–8 hr. Isolation of hybrids by Sephadex chromatography in 2 × SSC. Each value is the average of 2–3 determinations. The value for native DNA was determined by isolating the DNA by CsCl density gradient centrifugation after the Sephadex run.

RNase on the hybridized RNA, in agreement with the fact that the ratio 2′ to 3′ cytidylic acid was considerably lower than the value of 0.67 to be expected in the absence of any RNase action preceding the alkali hydrolysis.[8] When 2 × SSC was used as elution buffer for the Sephadex chromatography, there was a marked change in the base composition pattern, as illustrated by the lowering of the purine to pyrimidine ratio and by the apparent return to normal of the 2′ to 3′ cytidylic ratio. Under these conditions, however, the base ratios of the hybridized 28S HeLa RNA were still considerably different from those of the input RNA, in contrast to what was previously observed in the *E. coli* system.[1] This suggested that the observed differences were not due to secondary effect of RNase on RNA regularly and completely hydrogen-bonded with specific sites, but rather to the existence in the complexes being studied of a large proportion of partial hybrids involving DNA segments with base composition significantly different from that of ribosomal RNA. Figure 1 shows the sedimentation profile of the hybridized RNA as compared with that of the original RNA submitted to the same thermal treatment. In this experiment about 50 per cent of the hybridized RNA was recovered in the gradient as acid-insoluble material (as opposed to 100% recovery for the control RNA), the rest being represented by acid-soluble components in the upper part of the gradient. These acid-soluble products formed a peak separated from the meniscus and were not dialyzable against 2 *M* NaCl, findings which point to a size greater than pentanucleotides.[9] The acid-insoluble material is distributed over the whole gradient with a broad peak in the upper part and a long leading edge extending down to the bottom of the tube. An appreciable portion of the hybridized RNA consists of chains of sedimentation con-

TABLE 2

OVER-ALL NUCLEOTIDE COMPOSITION OF HELA 28S RNA HYBRIDIZED TO HOMOLOGOUS DNA

		Moles %								% C attacked by RNase
		A	C	U(T)	G	% GC	Pu/Pyr	G/A	2′C/3′C	
28S	Expt. 1	16.5	31.9	17.5	34.1	66.0	1.02	2.07	0.68	n.d.
28S	Expt. 2	16.4	31.3	17.3	35.1	66.4	1.06	2.14	0.66	n.d.
"Core" 28S RNA*		32.8	13.6	10.2	43.4	57.0	3.20	1.32	0.09	79.4
Hybridized 28S RNA (after RNase digestion)	Expt. 1†	14.2	12.2	7.4	66.2	78.4	4.10	4.66	0.36	33.8
	Expt. 2†	13.7	11.0	6.5	68.8	79.8	4.71	5.01	0.36	33.8
	Expt. 3†	20.8	16.7	11.9	50.6	67.3	2.49	2.43	0.67	n.d.
DNA		29.1	21.4	27.5	22.0	43.4	1.05	0.76		

* Isolated as described by Markham and Smith.[9]

† Conditions for separation of hybrid: expt. 1, 2 × RNase, TNC; expt. 2, 1 × RNase, TNC; expt. 3, 1 × RNase, 2 × SSC.

TNC: 0.01 *M* tris b. pH 7.4, 0.005 *M* NaCl, 0.0075 *M* Na citrate; 2 × SSC: 0.3 *M* NaCl, 0.03 *M* Na citrate; n.d.: nondetectable.

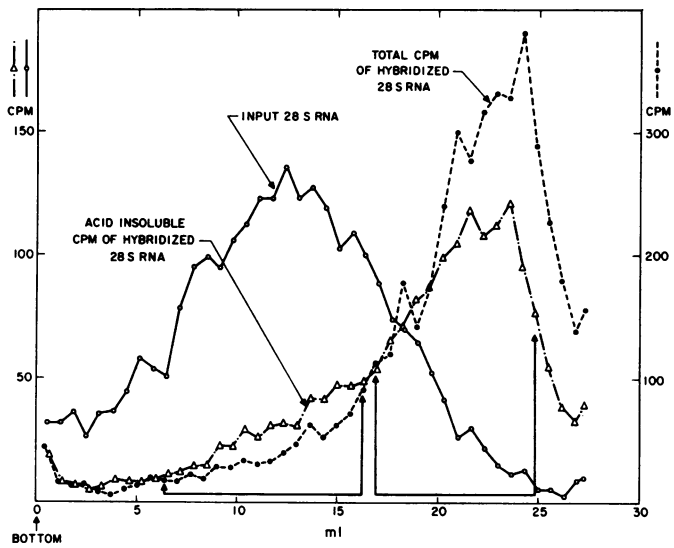

FIG. 1.—Composite diagram showing the sedimentation profile of the 28*S* P^{32} RNA hybridized with HeLa DNA as compared to that of the original RNA. One mixture containing 37.5 μg/ml DNA and 3.75 μg/ml 28S P^{32} RNA was incubated at 70°C for 8 hr. After slow cooling and RNase digestion (5 μg/ml, 21°C, 15 min), the mixture was run through Sephadex G-100 in 2 × SSC. The fractions containing DNA were pooled and filtered through nitrocellulose membranes (washed with 0.5 *M* KCl, 0.01 *M* tris, pH 7.4, at 53°C). The hybridized RNA was dissociated from DNA and run in a sucrose gradient for 40 hr at 24,000 rpm, 4°C, as described previously.[1] Fractions were collected from the bottom of the tube and analyzed for total and acid-precipitable (5% trichloroacetic acid with 200 μg bovine serum albumin as a carrier) P^{32} radioactivity. As a control, an aliquot of 28*S* P^{32} RNA was incubated at 70°C for 8 hr, then diluted with the buffer used for the elution of hybrid RNA from the membranes, subjected to the same thermal treatment employed in the dissociation steps, and run in a sucrose gradient. The H^3 RNA marker added to the elution buffer sedimented in identical position in the two sucrose gradients. The fractions of the hybridized RNA indicated by arrows were pooled and utilized for base composition analysis (Table 3).

stant similar to the average value for the original RNA. As shown in Table 3, while the base composition of the material in the light region of the gradient is considerably different from that of the input RNA and close to the one measured in the total hybridized RNA after isolation of the hybrid in 2 × SSC (Table 2), the RNA recovered in the heavy region has base ratios similar to those of the original RNA, the slight deviation observed possibly being attributable to contamination by slower sedimenting material. In the preceding paper evidence was presented concerning the invariance in size and base composition of the hybridized *E. coli* 23*S* RNA after repeated RNase digestion steps in 2 × SSC; on the other hand, no enzyme effect during and after dissociation of the hybrids has been detected on the H^3 control

TABLE 3

NUCLEOTIDE COMPOSITION OF HELA 28*S* RIBOSOMAL RNA HYBRIDIZED TO HOMOLOGOUS DNA, DETERMINED AFTER FRACTIONATION ACCORDING TO SIZE

Fractions of hybridized 28*S* RNA		Moles % A	C	U	G	Purines/ pyrimidines
Heavy region of sucrose gradient	Expt. 1	21.0	29.1	21.7	28.2	0.97
	Expt. 2	20.0	27.4	20.7	31.8	1.08
Light region of sucrose gradient	Expt. 1	21.9	15.7	19.9	42.4	1.80
	Expt. 2	19.2	17.0	17.7	46.0	1.88
Total acid-insoluble fraction		22.8	22.5	18.5	36.0	1.43

The material corresponding to expt. 1 came from the pooled fractions indicated by arrows in Fig. 1; the material corresponding to expt. 2 and the total acid-insoluble fraction came from two independent hybridizations.

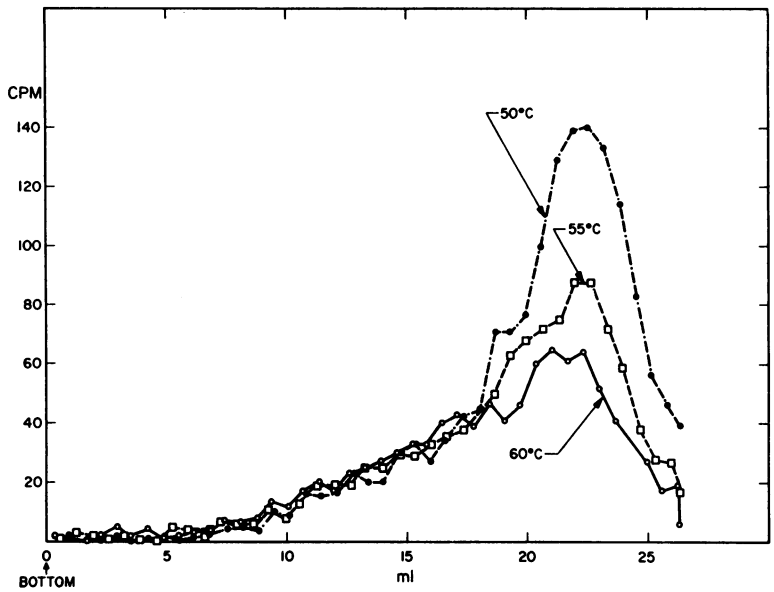

FIG. 2.—Effect of the temperature used in washing the nitrocellulose filters on the sedimentation profile of the 28S P^{32} RNA hybridized with HeLa DNA. One mixture containing 25 μg/ml DNA and 2.5 μg/ml 28S P^{32} RNA was incubated at 70°C for 8 hr. After RNase digestion and Sephadex chromatography, the fractions containing DNA were pooled and divided into three equal aliquots which were then filtered through nitrocellulose membranes and washed each with 120 ml KCl-tris at different temperatures. After dissociation of the hybrids, the hybridized RNA was run in sucrose gradients as described in Fig. 1. The recovered RNA plotted in this graph represents the acid-insoluble radioactivity.

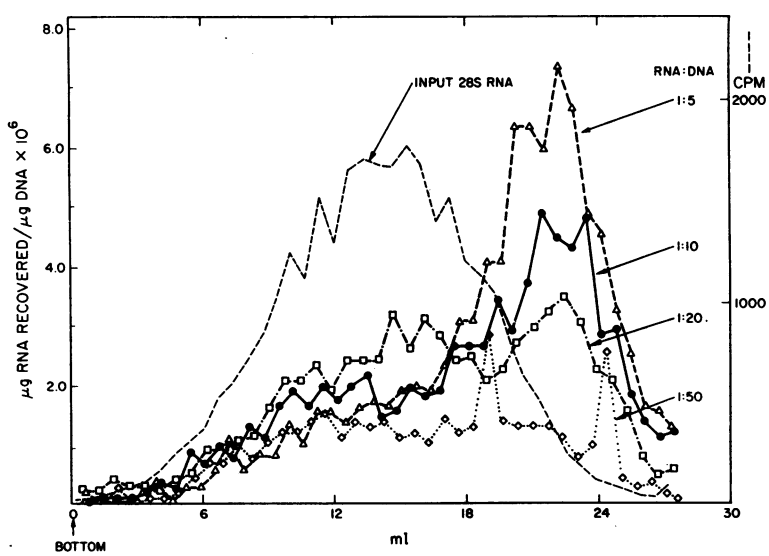

FIG. 3.—Composite diagram showing the sedimentation profile of the 28S P^{32} RNA hybridized with HeLa DNA at different RNA to DNA ratios, as compared to that of the original RNA. Four mixtures containing 16.7 μg/ml DNA and varying amounts of 28S P^{32} RNA were incubated at 70°C for $2^1/_2$ hr. Isolation of hybrids and sedimentation analysis of hybridized RNA as described in Fig. 1.

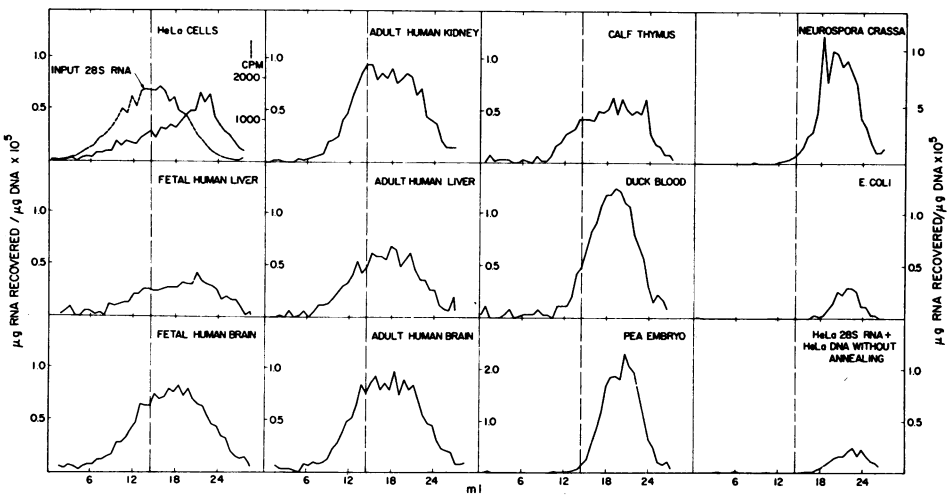

FIG. 4.—Sedimentation profiles of 28*S* P^{32} RNA hybridized with various DNA's. Each mixture contained 16.7 μg/ml DNA and 1.67 μg/ml P^{32} 28*S* RNA. Incubation at 70°C for $2^1/_2$ hr. Isolation of hybrids and sedimentation analysis of hybridized RNA as described in Fig. 1. All patterns are from expt. 2 analyzed in Table 4, except the RNA profile of the nonannealed mixture (expt. 1).

RNA present in the buffer used for dissociation. These observations indicate that the discrepancy in sedimentation pattern of the hybridized RNA from the input RNA does not result from RNase attack on longer polynucleotide stretches systematically hydrogen-bonded with DNA nor from enzyme effects on the dissociated RNA, but rather depends on the primary event of hybrid formation.

The study of the kinetics of formation of complexes involving longer RNA sequences showed that the process reaches a plateau after 2–3 hr incubation of the annealing mixture at 70°C.

Figure 2 shows that increasing the temperature of the buffer used for washing the nitrocellulose membranes from 50 to 60°C reduces the amount of the lighter acid-insoluble components without affecting the heavier components. The acid-soluble material decreases also approximately from 50 to 30 per cent. In the experiments described below, the temperature of 60°C was adopted for washing the nitrocellulose membranes.

As shown in Figure 3, while the amount of the lighter material augments with input RNA to DNA ratios increasing from 1:50 to 1:5, the heavier components reach a maximum level for ratios of RNA to DNA of 1:20 to 1:10, indicating a saturation of the corresponding sites (the slight decrease at a ratio of 1:5 is possibly the result of multiple occupancy of sites).

Figure 4 shows the sedimentation analysis of 28*S* RNA hybridized with various DNA's from human and heterologous sources. In these experiments 60–90 per cent of the eluted RNA was recovered as acid-insoluble radioactivity in the gradient, except in the hybridization with *E. coli* DNA and in the mixing experiment with HeLa DNA not involving annealing, where the recovery was, respectively, 40 and 21 per cent. A gradual broadening of the sedimentation profile toward the heavier side is evident going from *E. coli* to *Neurospora*, pea, duck, and mammals, an observation which is indicative of increasing degree of sequence

homology of the corresponding DNA's with HeLa 28*S* RNA. The portion of the sedimentation pattern which extends beyond the center of the profile of the input RNA is of the same order of magnitude for RNA derived from complexes with various human DNA's, while it is somewhat lower for RNA hybridized with calf thymus DNA and more so for that hybridized with duck DNA, becoming finally very small or nil in the case of RNA complexed with pea embryo or *E. coli* DNA. The spreading toward the heavier side of the RNA hybridized with *Neurospora* DNA appears to be the result of diffusion of the large peak of slower sedimenting components, rather than expression of the presence of a significant amount of heavier components.

The specificity pattern and the similarity in sedimentation properties and base composition to the original RNA suggest strongly that the heavier components in the gradient are derived from complexes involving ribosomal RNA sites. In Table 4 the fraction of various DNA's which presumably participates in specific hybridization with 28*S* RNA is indicated for two different cutoff lines selected in the control RNA sedimentation profile: each of these lines is arbitrarily chosen as the median of a symmetric distribution of heavy chains, with the portion of the individual pattern beyond the selected line representing the heavier half. At a cutoff delimiting the heaviest third of the control RNA distribution, about 3×10^{-5} of the HeLa DNA appears to be involved in specific hybridization with ribosomal RNA; a figure of the same order of magnitude ($3\text{–}5 \times 10^{-5}$) is obtained for other DNA's of human origin; the specificity pattern appears to be plausible at this cutoff.

On the basis of measurements made on RNA having the same thermal history as that used in the hybridization experiments,[1] the sedimentation constant of the heavier chains corresponding to the center of the input RNA profile has been estimated to be 7–8*S;* accordingly, their size would be 250–350 nucleotides. The slower sedimenting acid-insoluble components in the upper third of the gradient probably consist of 1–3*S* chains.

The hybridization of HeLa 18*S* with homologous DNA was investigated less extensively than that of the 28*S* RNA. The sedimentation analysis of 18*S* P^{32}

TABLE 4

FRACTION OF VARIOUS DNA'S INVOLVED IN HYBRIDIZATION WITH RNA CHAINS OF DIFFERENT SEDIMENTATION CONSTANT

	Cutoff at Limit of Heaviest Third of Control RNA Profile				Cutoff at Midpoint of Control RNA Profile			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2	
DNA source	RNA/DNA × 10^5	% Hybrid RNA	RNA/DNA × 10^5	% Hybrid RNA	RNA/DNA × 10^5	% Hybrid RNA	RNA/DNA × 10^5	% Hybrid RNA
HeLa	3.1	12	3.0	19	5.0	20	4.6	30
Adult human kidney			4.3	19			8.6	39
Adult human brain	3.2	20	4.6	25	7.4	46	9.5	48
Fetal human brain			4.8	26			8.6	46
Adult human liver	3.0	19	3.4	23	6.6	44	6.2	42
Fetal human liver	3.0	10	2.2	23	5.0	17	3.8	42
Calf thymus	2.0	9	2.2	15	4.2	20	4.1	28
Duck blood	0.5	0.9	1.0	5	3.8	7	2.4	12
Pea embryo	0.06	0.3	0.04	0.1	0.2	3	0.4	2
Neurospora crassa	0.8	1.3	0.6	0.6	2.2	4	3.0	3
E. coli	0.09	1.4	<0.01	<0.1	0.08	2	0.04	0.6
HeLa (without annealing)	0.06	0.8			0.08	0.8		

In expt. 1, incubation was at 70°C for 8 hr; in expt. 2, at 70°C for 2½ hr. See text for explanation of cut off points.

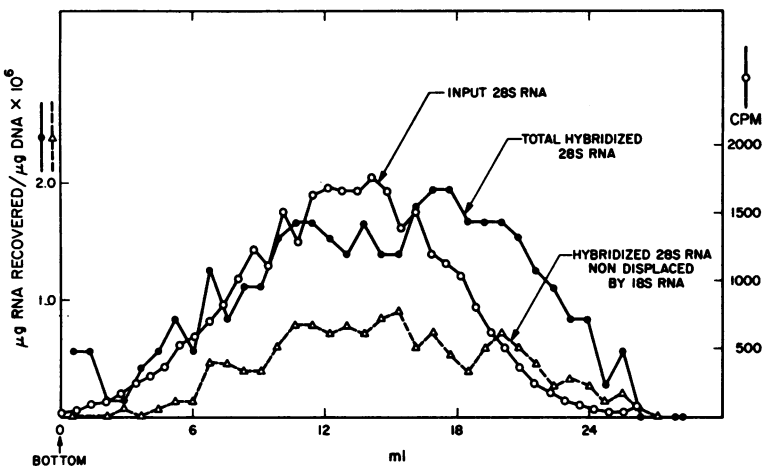

FIG. 5.—Composite diagram illustrating the sedimentation profile of 28*S* P^{32} RNA hybridized with HeLa DNA alone or in the presence of 20-fold excess of cold 18*S* HeLa RNA. One mixture containing 22.5 μg/ml DNA and 0.45 μg/ml 28*S* P^{32} RNA, the other containing 22.5 μg/ml DNA, 0.45 μg/ml 28*S* P^{32} RNA, and 9 μg/ml unlabeled 18*S* RNA. Both mixtures were incubated at 70°C for $2^1/_2$ hr. Isolation of hybrids and sedimentation analysis of hybridized RNA as described in Fig. 1. The over-all level of hybrid formed by P^{32} 28*S* RNA in the presence of 18*S* was 58% of the control (28*S* P^{32} RNA × HeLa DNA). In the same experiment, other incubation mixtures containing 20-fold excess of unlabeled 28*S* and *E. coli* 23*S* gave a level of hybrid which was, respectively, 16 and 94% of the control.

RNA hybridized with HeLa DNA showed also a considerable heterogeneity, with the heavier portion of the distribution sedimenting as fast as the input RNA; the sites corresponding to the heavier components appeared to be saturated at a level of 2–3 × 10^{-5} of the DNA.

Relationship between 28S and 18S RNA: While the presence of an excess of *E. coli* ribosomal RNA did not appreciably affect over-all complex formation between either P^{32} 28*S* or 18*S* RNA and HeLa DNA, the 28*S* and 18*S* competed considerably with each other (up to 60%). In order to test whether and to what extent this competition did actually involve long sequences specifically hybridized with presumptive ribosomal RNA sites, the sedimentation properties of the P^{32} 28*S* RNA hybridized with homologous DNA in the presence and absence of an excess of unlabeled 18*S* RNA were compared. As shown in Figure 5, the portion of hybridized 28*S* RNA which is not displaced by 18*S* has a sedimentation constant distribution similar to that of the total hybridized RNA.

Discussion.—The analysis presented in this paper has shown that a heterogeneous population of RNase-resistant segments can be recovered from hybrids between HeLa 28*S* and homologous DNA; in this population a fraction corresponding to 3–5 × 10^{-5} of HeLa DNA has sedimentation properties and base composition similar to those of the bulk of the input RNA, a result which points to a regular and complete hydrogen bonding of these chains with DNA. Such a finding, together with the specificity pattern shown by this portion of the complexes, suggests that their formation occurs at the level of specific ribosomal RNA sites. On the basis of the sedimentation properties of the hybridized RNA, a similar fraction (3–8 × 10^{-5}) of other DNA's of human origin appears to be involved in specific hybridization with 28*S* RNA. McConkey and Hopkins[10] have recently reported a

figure of about 5×10^{-5} for the fraction of HeLa DNA complementary to 28S RNA, as determined by measuring the over-all level of hybrid retained on Millipore filters after a disaggregation step at 60°C; although these results suggest a similar order of magnitude of the specific complexes, they are difficult to compare to ours for the difference in the conditions employed and because the nature of the hybrids involved was not analyzed. If the figures of 1.2×10^{13} daltons and 7×10^{12} daltons are used for the total DNA content of HeLa cells[10] and other human cells,[11] respectively, and if only one of the strands of DNA is transcribed *in vivo* into ribosomal RNA, as appears to be the case in bacteria,[1] it can be calculated that the DNA fraction involved in specific hybridization with 28S RNA would be equivalent to the sum of 200–400 stretches of the length of 28S molecules[12] in the HeLa genome and 150–350 stretches in other human genomes. These figures are only minimum estimates, because the absolute efficiency of hybridization is probably significantly lower than 100 per cent and because one fraction of the shorter regions of base pairing may actually involve ribosomal RNA sites. The fraction of DNA which appears to be capable of specific hybridization with 18S RNA is of the order of $2–3 \times 10^{-5}$ in HeLa cells; this would correspond to the sum of 400–600 stretches of the length of the 18S molecule[12] per cell. The cross-hybridization observed between the two RNA subclasses introduces, however, an uncertainty as to the fraction of DNA corresponding to 28S and, respectively, 18S sites. In spite of this uncertainty, the number of sites appears to be very large for both RNA species. This multiplicity, which has also been observed in bacteria,[1, 13] raises the problem of the variability and control of ribosomal RNA genes.

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