Hybridization of Messenger RNA with DNA from Plants*

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Received May 16, 1966

Natural DNA-RNA hybrids have been suggested in biological organisms (Spiegelman et al., 1961; Hayashi, 1965; Hayashi and Hayashi, 1966). The general consensus is that the complex consists of an RNA molecule bound to a complementary portion of the DNA strand. Artificial complexes between DNA and messenger RNA in microbial systems have been demonstrated (Hall and Spiegelman, 1962). Since nucleic acids of higher plants may be fractionated on methylated albumin kieselguhr (MAK) columns, it is possible to examine each RNA component in regard to its ability to hybridize with DNA in a manner similar to that for bacteria (Hall et al., 1964).

A great deal of recent work has been devoted to messenger RNA mainly, on its half-life and base composition (Ingle et al., 1965; Chroboczek and Cherry, 1966) in plants. Verification that the last fraction eluted from the MAK column is messenger RNA as judged by hybridization, was the major concern of this report.

Methods: Cotyledons were harvested from Virginia 56R peanuts (Arachis hypogaea L.) after the seeds had been germinated on moist paper in the dark for 2 days. Hybridization tests were conducted with unlabeled DNA and P^{32}-labeled RNA. Unlabeled DNA was extracted from cotyledons without incubation and subsequently purified on a MAK column. Labeled RNA was extracted from cotyledons that were first incubated for 3 hours with P^{32} in a medium containing 1% sucrose, 10^{-4} M ammonium citrate buffer, pH 6. Procedures for phenol extraction and MAK column chromatography of the nucleic acids have been described (Cherry et al., 1965). The nucleic acids were fractionated into six RNA components, which were eluted from the column in the following order: I, first soluble; II, second soluble;

*This investigation was supported by a U.S. Atomic Energy Commission contract, COO-1313-11. Journal paper No. 2671 of the Purdue Agriculture Experiment Station.

III, DNA-RNA; IV, light ribosomal; V, heavy ribosomal and VI, messenger RNA. Nucleic acid components were collected from the peaks as indicated by their optical density (260 mμ) readings and radioactivity measurements. They were precipitated in ethanol, dialyzed against 0.05 M phosphate buffer pH 6.7 and then filtered through cellulose powder in sintered glass funnels to eliminate any protein that might have eluted from the MAK column with the nucleic acids (Gillespie and Spiegelman, 1965). The DNA was collected from component III (DNA peak) and after dialysis it was heat denatured at 90°C for 10 minutes. Cocklebur (Xanthium pensylvanicum Wall.) DNA (unlabeled) was obtained from leaves in a way analogous to that for the nucleic acids from apical bud tissue as described previously (Cherry and van Huystee, 1965a). Annealing of the DNA and RNA was done according to the procedures of Giacomoni and Spiegelman (1962). The annealing process was divided into 3 temperature periods beginning with 16 hours at 40°C, followed by 2 hours at 70°C and then a gradual cooling over approximately 5 hours to 40°C at which temperature the mixtures were kept for another 16 hours. The mixture was incubated with 0.2 ml pancreatic RNAase (10μg/ml) for 30 minutes at 30°C to remove the non-hybridized RNA. The hybrid complex after cooling was finally precipitated with 10% TCA in the presence of 50 μg/ml of carrier DNA. The precipitate was collected on a nitrocellulose (Bac-T-Flex, type B) filter and washed with 10% TCA; the radioactivity was measured in a Tricarb liquid scintillation spectrometer. Two control samples were carried along with each experiment. In both samples, water was substituted for DNA in the annealing medium; one was not digested with RNAase while the other was. The first gave a total activity of all P^{32}-RNA added and the second the minimum counts (noise or background), which were then used to calculate the total hybridizable counts. In some experiments an annealed sample was used for CsCl density gradient centrifugation as outlined previously (Cherry, 1964).

Results and Discussion: Increasing the NaCl concentration from 0.2 M to 1.5 M in the annealing medium increased the percent of hybridization several fold (Fig. 1), which agrees with data of Hall et al. (1964) for microbial systems. It was decided to use 0.8 M NaCl in 0.01 M Tris, pH 7.3 for all further work in this paper. A saturation curve was obtained by varying the ratio of DNA to RNA from 0.25 to 2.0 (Fig. 2). It appears that this curve reaches a plateau when the ratio is near one. Equal amounts of DNA and messenger RNA were used in the remaining experiments. In the case of bacteria the saturation point of DNA to RNA is more than 10. However, higher plants might produce a greater variety of messenger RNA and therefore require more of it to saturate DNA than is the case with bacteria.

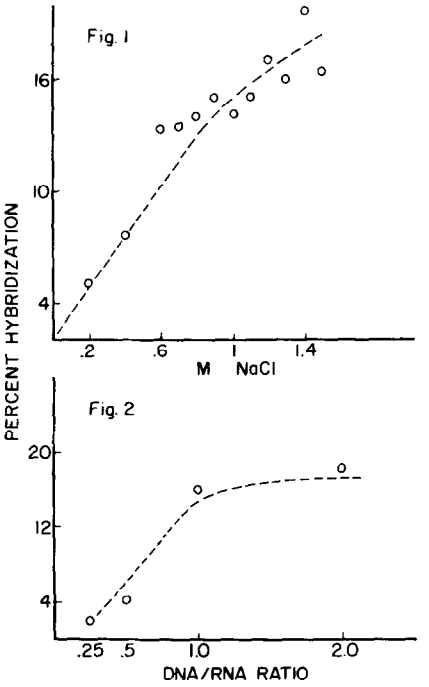

Fig. 1. Percent of DNA-RNA hybridization with increasing NaCl concentration in 0.01 M Tris buffer pH 7.3. Except for Fig. 2, 40 µg of DNA and messenger RNA each was used in all cases.
Fig. 2. Percent hybridization as influenced by increasing DNA. Messenger RNA was kept constant at 40 µg.

The five (omitting component III) RNA components obtained from MAK columns as mentioned above were annealed with homologous DNA. Their degree of hybridization fluctuated considerably as shown in Table I. The fraction termed messenger RNA hybridizes with homologous DNA three times greater than any other RNA component. The ease of messenger RNA to unite with homologous DNA suggests that this RNA component is in fact messenger RNA.

To determine whether the nucleic acid component used for hybridization (Table I) was in fact RNA, each fraction was incubated with 0.5 M KOH at 37° for 16 hours. The radioactivity recovered in the supernatant is expressed as percent of total CPM for each fraction in Table 2. If the non-hydrolysable material accounts partly or totally for some of the RNAase resistant radioactivity (Table I), it would not nullify the amount of hybridization for messenger RNA but it might for ribosomal and soluble RNA.

Table 1

Hybrid formation between RNA and DNA from peanut cotyledons

RNA fraction* from MAK column	Counts per min. annealed			Percent hybridized
	Total		Noise	
soluble$_1$	439	54	37	3.9
soluble$_2$	1841	137	92	2.4
light ribosomal	2937	339	235	3.5
heavy ribosomal	8317	470	326	1.7
messenger	16468	7029	5142	11.4
purified messenger	1008	590	435	15.4

*The amounts of RNA used were 30 µg for soluble$_1$, soluble$_2$ light ribosomal; 80 µg for heavy ribosomal; and 40 µg for messenger and purified messenger. DNA was obtained from 2 day old, non-incubated peanut cotyledons and purified on a MAK column.

Table 2

Radioactivity recovered from each RNA fraction after KOH hydrolysis.*

Nucleic acid fractions	Counts per min.		Percent Hydrolysed
	Total	Hydrolysable	
soluble	814	766	96
DNA-RNA	2370	1156	50
light ribosomal	2221	1980	90
heavy ribosomal	16828	16000	95
messenger	11866	11004	94

*Nucleic acids from P^{32} labeled peanut cotyledons were fractionated on a MAK column. Each of the five fractions indicated were precipitated in ethanol and dialyzed; subsequently, aliquots of each were incubated in 0.5 M KOH at 37°C for 16 hours or longer.

There were in general high noise levels in these experiments due to incomplete digestion of non-annealed labeled RNA. It has been impossible to lower these levels. In the case of plant RNA, it may be that the "core" remaining after digestion with pancreatic RNAase (Anfinsen and White, 1961) is collected on the membrane filter and thus increases the noise.

The possibility that the P^{32} labeled RNA from peanut cotyledons was contaminated with bacterial RNA was also considered. During this study peanut seeds were treated with 0.1% sodium hypochlorite (NaOCl) for $1\frac{1}{2}$ min. before germination. NaOCl treatment reduced the bacterial population from 6,000 - 8,000 colonies per cotyledon for non-treated seeds to 3,000 - 5,000 colonies per cotyledon.** The incorporation of P^{32} into the nucleic acids did not change by this treatment. Also the labeling patterns for the nucleic acids fractionated on MAK columns were identical. However, chloramphenicol (20 µg/ml) as a bacteriostatic agent (Wilson, 1966) reduced the P^{32} incorporation by 50% in the light and heavy ribosomal RNAs but did not noticeably effect the messenger RNA, which agrees with previous work in this laboratory (Carpenter and Cherry, 1966). In these experiments actinomycin D (10 µg/ml) inhibited P^{32} incorporation into the ribosomal fractions by 70% but the synthesis of messenger RNA was inhibited only 35%. Labeled messenger RNA fractions obtained from control and treated seeds were isolated as described above and used for hybridization tests with DNA extracted from dry seeds (unimbibed). The results are shown in Table 3.

Table 3

Effect of bacteriostatic agents on hybridization of messenger RNA

Treatment	Counts per min. Total	Annealed	Noise	Percent Hybridized
Control	1,439	828	660	12
NaOCl (0.1%)	1,771	542	340	11
Chloramphenicol (20 µg/ml)	1,103	698	578	11
Actinomycin D (10 µg/ml)	859	389	358	4

**Grateful acknowledgement is made here for the assistance received from Drs. V. Rodwell and H. S. Ragheb, Biochem. Department, Purdue University.

The percent hybridization of the control did not differ from that in Table 1. Even though the NaOCl treatment reduced the bacterial population about 50%, no large increase in the percent hybridization was observed. Similarly in the case of messenger RNA from chloramphenicol treated tissue hybridization was slightly lower instead of higher which would not have been the case if there was a dilution of plant messenger RNA with bacterial RNA. Actinomycin D reduced the percent hybridization severely, probably due to the interference with the normal synthesis of messenger RNA (Chroboczek and Cherry, 1966). From our present studies it seems that bacterial contamination does not play a major role in the synthesis of various nucleic acid fractions of the peanut cotyledons.

The remaining research dealt only with the messenger RNA component of peanut cotyledons. The close proximity of the heavy ribosomal RNA component (low hybridization) to the messenger component as it is eluted from the MAK column might contaminate the messenger component with some low hybridizable RNA. The messenger component was therefore purified by rechromatography (Cherry and van Huystee, 1965b) and a higher percent hybridization was noted (Table 1). If a sample of the DNA-RNA hybrid was fractioned by CsCl gradient centrifugation, (Fig. 3) the greatest amount of radioactivity peaked with the unlabeled DNA (O.D. 260) peak. This corroborates the previous data that a hybrid was indeed formed.

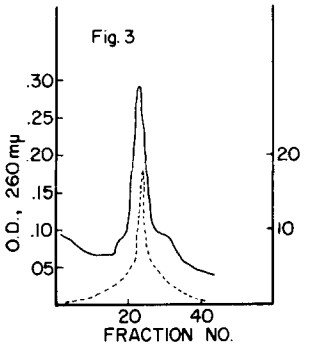

Fig. 3. A CsCl centrifugation gradient profile of an artificial hybrid. RNA and DNA were annealed as described in the text.

Finally the binding capacity of messenger RNA with homologous DNA and with heterologous DNA was determined. The heterologous DNA from cocklebur or from calf thymus (highly polymerized purchased from Sigma Chemical Co.) were annealed with messenger RNA from peanut cotyledons. The data from Table 4 indicates clearly the specificity of messenger RNA and homologous DNA. Furthermore, there is a greater specificity of peanut RNA for cocklebur DNA than for animal DNA.

Table 4

Hybrid formation between messenger RNA of peanut cotyledons with homologous and heterologous DNA*

DNA source	Total counts	Hybridizable counts	Percent hybridized
Peanut	1317	134	10.1
Cocklebur	1118	62	5.5
Calf thymus	1245	40	3.2

*The amounts of DNA and RNA used were 40 µg each in all trials. Counts are corrected for background.

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