A METHOD FOR THE DETECTION OF RNA-DNA COMPLEXES

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Although several methods have been developed to study the interaction between DNA and RNA (Hall and Spiegelman, 1961; Bautz and Hall, 1962; Bolton and McCarthy, 1962), none of them are suitable for detailed kinetic studies. In an attempt to find a faster and more quantitative method we discovered that nitrocellulose membrane filters have absorption characteristics that can be used for the separation of free RNA from RNA bound to DNA. In salt solutions of moderate concentration these filters absorb heat-denatured DNA as well as complexes between DNA and RNA, whereas free RNA passes through the filter. Thus the reaction of labeled RNA with unlabeled DNA can be followed by measuring, in samples taken at different times, the amount of radioactivity caught on the filter.

MATERIALS AND METHODS

Nitrocellulose Membrane Filters:--The filters used were Type A, coarse, for aqueous solutions, 27 mm in diameter, and were obtained from Schleicher and Schuell, Keene, N.H.

RNA:--A culture of T2-infected E. coli was chilled to 0°C, centrifuged, resuspended in 1/50 the original volume of 0.01 M $Mg(OAc)_2$, 0.06 M KCl, 0.01 M Tris (pH 7.3), and frozen. After thawing, the suspension was stirred 3 min at 50°C, acidified with 1/40 volume 0.1 N HOAc, and then subjected to the hot phenol purification procedure (Scherrer and Darnell, 1962). After the last phenol extraction, the RNA solution was passed through a column of Sephadex G-25 (coarse, equilibrated with 0.01 M KOAc-HOAc buffer, pH 5.2). After passage through the column,

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the RNA was adjusted to pH 7.3, 0.5 M KCl, and filtered through nitrocellulose filters, one filter per mg RNA. This step removes phosphorous-containing impurities which have a high affinity for nitrocellulose.

DNA:--DNA was purified from phage T2 and T4 by the phenol extraction procedure (Grossman, Levine, and Allison, 1961). It was denatured by heating at 100°C for 15 min in 0.06 M KCl, 0.01 M Tris, pH 7.3, at concentrations varying from 0.6 to 150 μg/ml. The hot DNA solution was cooled by pouring directly into 1/3 volume ice, made from the same solvent, at -30°C.

ABSORPTION BEHAVIOR OF NUCLEIC ACIDS ON NITROCELLULOSE MEMBRANE FILTERS

RNA:--Membrane filters do not appreciably absorb RNA in solutions containing 0.2 to .85 M KCl (Fig. 1). By washing with 60 ml of 0.5 M KCl, 0.01 M Tris, pH 7.3 absorption of RNA is further reduced to about 0.5%.

Heat-Denatured DNA:--The absorption of denatured T2 DNA by membrane filters is essentially complete over a wide range of salt concentration (Fig. 1). The quantity of radioactive DNA absorbed is proportional to the amount of DNA filtered down to the smallest amount measured (Fig. 2). The proportionality extends upward to about 200 μg DNA per filter. Above 100 μg DNA per filter, filtration becomes very slow.

Native DNA:--Native T4 DNA preparations are heterogeneous with respect to absorption on nitrocellulose. The fraction absorbed varies from preparation to preparation (10-50%), being least in the most extensively purified preparations.

The DNA fraction which passes through the filter shows no detectable absorption when filtered a second time. Upon heating, this DNA is rendered absorbable. The relation between temperature of heating and percent of DNA absorbed (Fig. 3) approximates the thermal denaturation curve of DNA. In SSC buffer the midpoint of the transition is 85°C, as measured by either filtration or hyperchromism (Marmur and Doty, 1959).

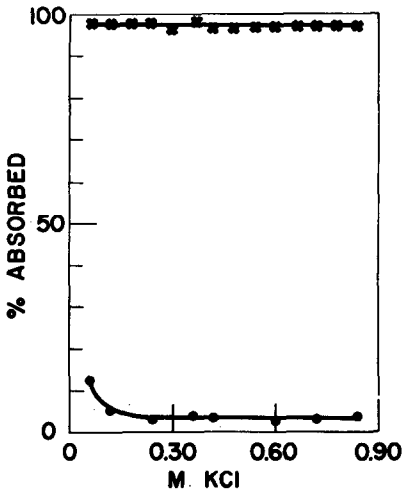

Figure 1.

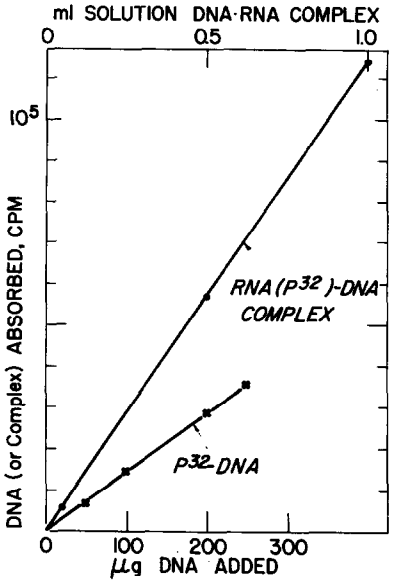

Figure 2.

Fig. 1. Absorption of denatured DNA, X———X, and RNA, ●———●, on nitrocellulose membrane filters. To each filter were added 2.5 ml of a solution containing: 89 μg heat-denatured unlabeled T4 DNA; 9.0 μg heat-denatured P^{32} T2 DNA, 40,000 cpm/μg; 43 ug H^3 T2 RNA, 680 cpm/μg (labeled from 0-5' after T2 infection); KCl was added to give the indicated concentration. All samples were buffered with 0.01 M Tris, pH 7.3. The filters were not washed as in the regular procedure.

Fig. 2. Absorption of denatured DNA and of DNA-RNA complex on nitrocellulose membrane filters in 0.5 M KCl. DNA was a mixture of T4 DNA and T2 DNA (P^{32}, 35,000 cpm/μg, in the ratio 250/1. The DNA-RNA complex was formed from heat-denatured T2 DNA, (24 μg/ml) and 15-19' P^{32}-T2 RNA, 14.1 μg/ml, 13,000 cpm/μg, incubated for 70 min. at 65-67°C in 0.5 M KCl, 0.01 M Tris, pH 7.3.

RNA-DNA COMPLEX FORMATION

When incubated with denatured T2 DNA in saline solution, T2-specific RNA is converted to a form which is absorbed by membrane filters (Fig. 4). Several lines of evidence suggest that this change in absorption behavior is a consequence of RNA-DNA complex formation:

1. The process requires elevated temperature (>40°C) and moderately high ionic strength (>0.1 M), as do the recombination of complementary DNA

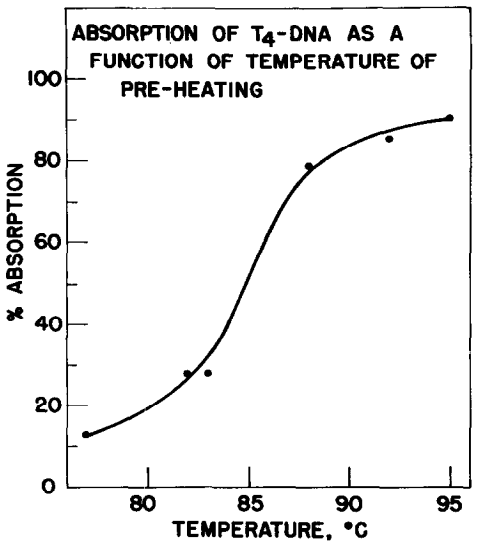

Figure 3.

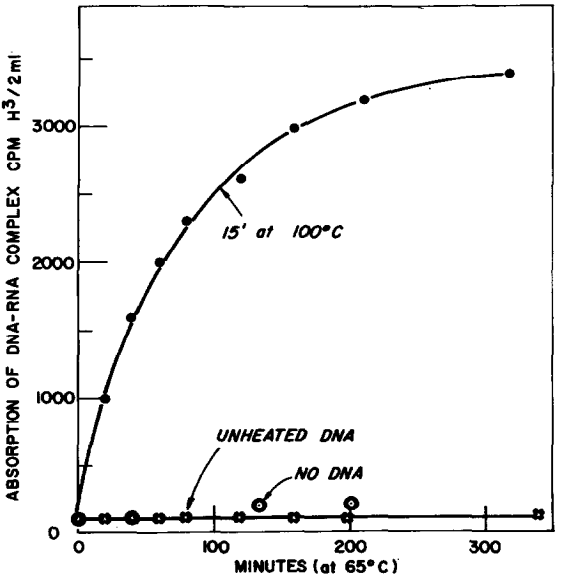

Figure 4.

Fig. 3. Absorption of T4 DNA on nitrocellulose membrane filters after heating the DNA to various temperatures. T4 DNA, which had been passed through membrane filters in order to remove all absorbable DNA, was diluted into 0.15M NaCl, .015 M Na citrate buffer to give a final concentration of 53 μg/ml DNA. After heating for 15 minutes at the indicated temperature the solution was fast cooled and diluted to give 24 μg/ml DNA in 0.5 M KCl, 0.01 M Tris, pH 7.3; 4 ml of this solution were added to the filter. The absorption to the filter was computed from the extinction of the filtrate at 260 mμ.

Fig. 4. The Dependence of RNA-DNA complex formation on denatured DNA.
X——X, 0-5' H^3 T2 RNA, 5.2 μg/ml, 680 cpm/μg; native T4 DNA, 20 μg/ml, 45% of which was absorbed on the filter in the native state. ●——●, DNA heat-treated for 15 minutes at 100°, otherwise as X——X; ◉——◉, H^3 T2 RNA, 5.2 μg/ml, 680 cpm/μg.

strands (Marmur and Lane, 1960) and the formation of RNA-DNA hybrids (Hall and Spiegelman, 1961). In 0.5 M KCl, the rate of complex formation, as measured by the filtration technique, is maximal at 67°C.

2. Incubation of T2-specific RNA under these conditions produces no filter-absorbable RNA unless heated T2 DNA is present (Fig. 4). Although 50% of the native DNA preparation tested was absorbed, RNA was not complexed by it.

3. Over a 100-fold range of the concentration of each reactant, the rate at which RNA becomes absorbable is proportional to the product (RNA concentration) x (DNA concentration).

In order to determine whether the RNA-DNA complexes absorbed by membrane filters are of a specific nature, heterologous RNA-DNA mixtures were tested. Pulse-labeled RNA from T2 infected E. coli readily formed complexes with denatured DNA from either T2 or T4, but did not react detectibly with DNA from E. coli, B. cereus, B. subtilis, or calf thymus. (Denatured DNA from each of these sources is efficiently absorbed by the filter). Further evidence for specificity is the fact that complex formation, filtering, and elution of the complex permits the isolation of T4-specific RNA of essentially 100% purity, as judged by its nucleotide composition (CMP--15%, AMP--32%, UMP--31%, GMP--21%).

DETECTION OF RNA-DNA COMPLEXES BY FILTRATION

Removal of Free RNA:--The major problem in using absorption on nitrocellulose to detect RNA-DNA complexes has been to reduce the "background" caused by trapping of free RNA and/or other labeled substances. This background has been reduced to 0.5% (0.5% of the labeled RNA passing through a filter is caught) by adopting the following measures: soaking the filters in KCl solution prior to use (soaking in water is not effective), extensive washing of the filter after absorption, and filtration of the RNA prior to complex formation (see Materials and Methods). Some further decrease in RNA absorption can be obtained by washing at higher temperature (60°C).

Reproducibility of the Method:--When the foregoing precautions are observed, there is both reproducibility between replicate samples and strict proportionalit between the quantity of RNA-DNA complex in the sample and the radioactivity of th filter (Fig. 2). When conditions are such that the fraction of labeled RNA complexed is small (uniformly labeled RNA, or high RNA/DNA ratio), detection of complexes may be made difficult by the variable background caused by free RNA absorption. In such circumstances, proportionality of the results obtained with

equivalent samples of varying size provides a useful criterion for distinguishing legitimate complex formation from RNA "noise".

Absorption-Elution Properties of Complexes:--Absorption of RNA-DNA complex to the filter from 0.5 M KCl solution is quite insensitive to pH in the range $9 > pH > 5$.

At pH 7 there is no elution of the complex at room temperature even when 200 ml of the KCl solution is passed through the filter; elution starts to occur only above 60°C. If the KCl concentration is lowered to 0.05 M, absorption of the complex is incomplete even at room temperature. RNA-DNA complex already absorbed on a filter can be eluted without dissociation by passing through the filter 2 ml of 0.01 M Tris, pH 7.3. The elution is more complete if the filter has been soaked only a few seconds before use.

Detailed Procedure for Routine Analysis:--The sample, which may vary from 10 µl to 5 ml depending upon the radioactivity of RNA, is diluted into 15 ml of 0.5 M KCl, 0.01 M Tris, pH 7.3, at room temperature. In the diluted state at this temperature no further reaction occurs and the hybrid is stable for at least 24 hours; thus the sample can be filtered whenever time permits, and sampling can be done at short intervals (20 seconds or less).

The filter is soaked in the KCl solution for at least 10 min before use, and approximately 10 ml of the same solution is passed through the filter before the sample is applied. Suction is provided by an aspirator, giving a vacuum of approximately 15 mm Hg. The filter is mounted on a stainless steel grid with a stainless steel cylinder placed on top as described by Roberts et al. (1957). The filter is finally washed with 60 ml of the KCl solution at room temperature, dried, placed in a vial with scintillating solution, and counted.

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