INTERFERENCE OF SYMMETRICALLY TRANSCRIBED RNA IN THE HYBRIDIZATION COMPETITION EXPERIMENT

A re-evaluation of the nitrocellulose filter technique for detecting RNA–DNA hybrids

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Received 8 April 1980
Revised version received 12 May 1980

1. Introduction

RNA–DNA hybridization experiments in the presence of competitor RNA have been used to demonstrate sequence similarities between RNA populations and to establish the identity of RNA molecules in many different biological systems. In the T_4 bacteriophage system, hybridization analysis of RNA synthesized in infected cells has provided detailed information about the control of transcription. Comparison of the RNA populations present at early and late times suggest that these sequences are transcribed from different genes and that each of the early and late classes consists of two subclasses [1–5]. Using separated T_4 DNA strands in the hybridization experiments above, early and late messengers appeared to be transcribed largely from different strands of the genome. Furthermore, some regions on this phage genome seem to be symmetrically transcribed [6], since the labelled RNA expressed at one time (e.g., early) was not degraded by RNase after annealing with unlabelled RNA isolated at another time (e.g., late) after infection. Further, sucrose gradient analysis and hydroxyapatite chromatography of complementary RNA have shown that the complementary RNA represents regions on larger RNA molecules [7].

Since both the assay for symmetrical RNA and the hybridization-competition assay involve annealing with a low concentration of labelled RNA and a high concentration of unlabelled RNA it was of interest to determine the extent of formation of double-stranded RNA in the competition assay. The interference RNA–RNA duplex formation in competition experiments has been mentioned in [8], but direct measurements of such formation in this particular reaction have not been reported.

The different products formed in the hybridization-competition experiment with T_4 RNA molecules have been analyzed by digestion using various nucleases as well as by isopycnic centrifugation in Cs_2SO_4 gradients. The data show that a considerable amount of double-stranded RNA is formed at high concentrations of competitor, and also that some perfect hybrid is lost in the nitrocellulose filter assay commonly used. The formation of this double-stranded RNA as well as the formation of perfect hybrid may interfere with the evaluation of the results from the competition experiments.

2. Materials and methods

Escherichia coli B was a gift from Dr B. D. Hall and the bacteriophage T_4 used in this study was from Dr O. Skøld. [5-^{3}H]Uracil was 47 Ci/mmol and was purchased from The Radiochemical Centre, Amersham. Electrophoretically purified DNase I, pancreatic RNase, T_1-RNase, Trizma Base and amylase powder (from *Aspergillus oryzae*) were from Sigma. The other chemicals used were of the highest purity commercially available. S_1-Nuclease was prepared from amylase-powder according to [9] with the omission of Sephadex G-100 gel filtration.

In vivo RNA from T_4-infected cells was prepared from cells grown in M-9 medium [10] at 30°C. The multiplicity of infection used was ~10. RNA was extracted using the hot phenol method [2]. Labelled RNA was obtained from cells pulse-labelled 20–30

min after infection with 400 μCi/ml [5-^{3}H]uracil. Purified RNA had spec. act. 50 000–80 000 cpm/μg RNA.

Native double-stranded unlabelled DNA from purified T_4 phage was isolated by the procedure in [11].

The hybridization-competition analysis was done as in [1]. Annealing took place in 0.5 M KCl 0.01 M Tris–HCl (pH 7.3, 25°C) at 67°C for 3 h. The hybrids were then treated with T_1-RNase, pancreatic RNase, DNase I, S_1-nuclease, or mixtures of these enzymes depending on the method of detection used and the purpose of the competition-analysis. Unlabelled single-stranded RNA was degraded with 25 μg/ml pancreatic RNase, 30 U/ml T_1-RNase in 10 mM Tris–HCl (pH 7.3, 25°C) and 0.35 M KCl. RNA in hybrids was completely digested if 200 μg/ml DNase I and 10 mM $MgCl_2$ were added to the RNase mixture. Incubation was at 37°C for 30 min.

When only RNase treatment was employed, the hybrids were collected on presoaked nitrocellulose filters, washed with hybridization buffer, dried in a vacuum-oven at 80°C for ≥45 min and assayed for radioactivity in a scintillation system.

When joint RNase-DNase or S_1-nuclease treatment was used, a fixed volume was applied to a 13 mm glass filter and immediately soaked in 10% ice-cold trichloroacetic acid. After a minimum of 15 min the filters were transferred to filtration equipment and washed with 2 ml 10% trichloroacetic acid, followed by 10 ml of an ethanol–ether (1:1) mixture. The filters were then dried and assayed for radioactivity.

To analyse the hybridization-competition products by isopycnic centrifugation in the presence of Cs_2SO_4, the hybridization reaction was stopped in ice and the samples were diluted with 1 vol. 2 × S_1-buffer (S_1-buffer: 0.03 M NaAc, 0.05 M NaCl, 1 mM $ZnSO_4$, 5% glycerol, pH 4.6) and 0.5 vol. 1 × S_1-buffer containing 0.3 M NaCl. S_1-Nuclease was then added to a concentration known to digest all the single-stranded nucleic acids present. The mixtures were incubated for 90 min at 45°C, then Cs_2SO_4 was added to a final density of 1.5800 g/ml. The gradients contained 0.1 M NaCl, 0.05% Sarcosyl, 2 mM EDTA and 0.01 M Tris–HCl (pH 7.3, 25°C), and were run in an SW-50.1 rotor at 40 000 rev./min for 70 h at 8°C. The gradients were then harvested from the bottom of the tubes, the fractions were precipitated with trichloroacetic acid and assayed for radioactivity.

3. Results

To determine the influence of RNA–RNA duplex formation in the hybridization experiment, we have compared three different ways of measuring the amount of radioactivity present as hybrid in the presence of competing unlabelled RNA.

When using the classical nitrocellulose filter technique to collect the RNA–DNA hybrids ≤51% of the radioactivity of late pulse-labelled RNA could compete from hybrid formation by early unlabelled RNA whereas late unlabelled RNA gives complete competition, as expected (fig.1). This experiment was one of the first to show that the development

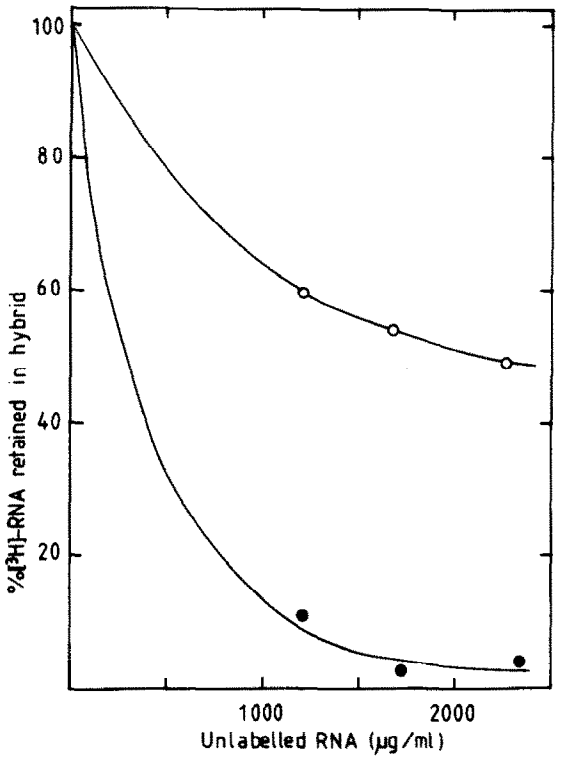

Fig.1. Hybridization-competition of 20–30 min labelled T_4 RNA and heat-denatured T_4 DNA with 6 min or 30 min unlabelled T_4 RNA. The annealing was carried out in 0.09 ml at 67°C for 3 h. T_4 DNA was 22.8 μg/ml and labelled RNA 1.78 μg/ml (90 000 cpm/μg). The hybridization reaction was stopped by diluting 1:1 with 10 mM Tris–HCl (pH 7.3, 25°C) then treating the mixture with nucleases as in section 2. The RNase-treated hybrids were then collected by filtration on nitrocellulose filters. 6.9% or 1000 cpm of the input RNA was resistant to RNase in the absence of competitor; (○——○) competition with 6 min unlabelled RNA and (●——●) with 30 min unlabelled RNA.

of T_4 is a temporal event [1] and that the process is regulated at the transcriptional level. Since then similar but more refined competition experiments have been set up and have resulted in a division of the T_4 RNA populations into at least four different classes [2,3]. By this method only radioactive RNA annealed to longer stretches of single-stranded DNA is retained on the filter since the binding is specific for single-stranded DNA [13], and neither perfect hybrids [14] nor double-stranded RNA (not shown) nor single-stranded RNA [13] bind to the filter under the conditions used.

Fig.2A shows the amount of trichloroacetic acid-precipitable radioactivity recovered after treating the hybridization mixture with RNases to degrade single-stranded RNA. The radioactivity retained on the filters in this way should be the sum of radioactivity found as RNA–DNA complexes since these are resistant to RNases. As can be seen in fig.2, with unlabelled 6 min RNA as competitor the curve levels off at 92%, and if we assume that the DNA hybrid accounts for 51% then the remaining 41% of the RNase-resistant trichloroacetic acid-precipitable material should represent RNA–RNA complexes. This equals 1271 cpm or 8.8% of the total input radioactivity which corresponds well to the amount of symmetrical RNA found under similar conditions [6,15]. With 30 min RNA as competitor, the competition curve levels off at 21%. This equals 649 cpm or 4.5% of the input. This is a high percentage of symmetrical RNA compared to what is obtained as double-stranded RNA when annealing late pulse-labelled RNA with unlabelled late RNA [6,15].

After annealing, the hybridization-mixture was treated with both RNases and DNase I, to measure directly the amount of RNA–RNA duplexes formed; RNA–DNA hybrids are sensitive to DNase I and therefore the trichloroacetic acid-precipitable material obtained should represent only double-stranded RNA.

Fig.2B shows that the amount of double-stranded RNA formed when 6 min unlabelled RNA is used

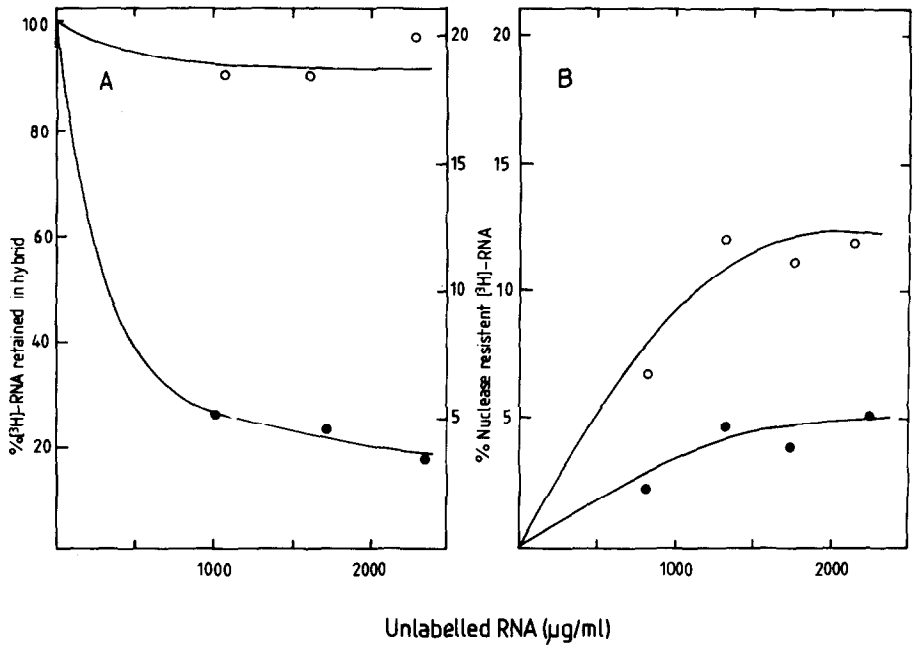

Fig.2. Hybridization-competition of 20–30 min labelled T_4 RNA and heat-denatured T_4 DNA with 6 min or 30 min unlabelled T_4 RNA. The annealing conditions were the same as those in fig.1. (A) After treatment with RNases the samples were precipitated with trichloroacetic acid on glass filters. 21.5% or 3100 cpm were resistant to RNases after annealing in the absence of competitor. The higher recovery here compared to that obtained in fig.1 is due to a higher counting efficiency when the hybrid is collected on glass filters. (B) The hybridization solution was treated with DNase I in addition to RNases as in section 2 prior to precipitation with trichloroacetic acid. (o——o) Competition with unlabelled 6 min RNA; (•——•) competition with 30 min unlabelled RNA.

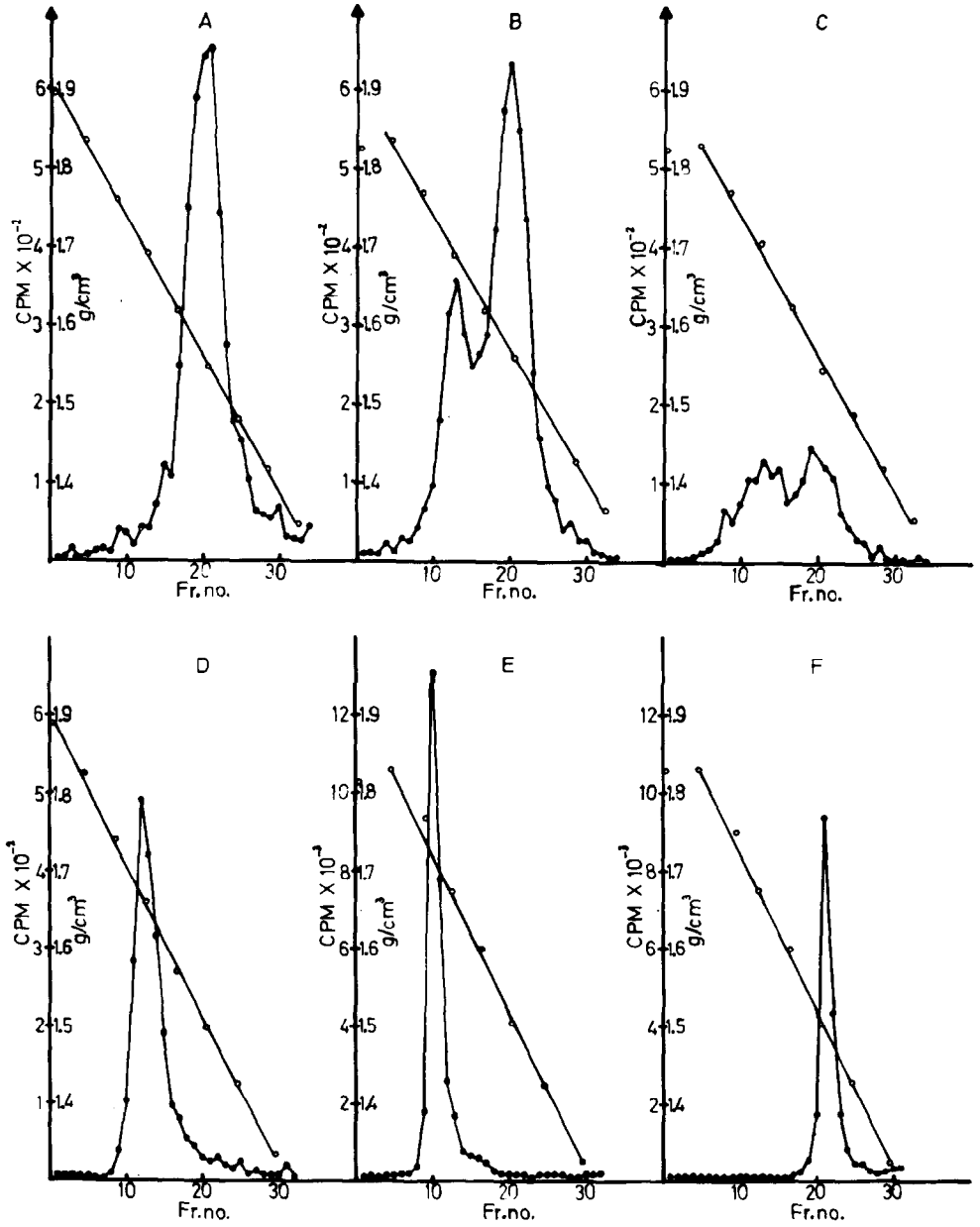

Fig.3. Isopycnic centrifugation of S_1-nuclease treated hybridization-competition mixtures in Cs_2SO_4. The hybridization-competition was done as in fig.1. After completion of the annealing reaction the mixtures were treated with S_1-nuclease (section 2). (A) Without competitor; (B) with 6 min RNA, 2335 μg/ml; (C) with 30 min RNA, 2365 μg/ml; (D) 8500 cpm isolated RNA–RNA complex; (E) 0.78 μg/ml 20–30 min [^{3}H]T_4 RNA (90 000 cpm/μg); (F) 0.08 μg [^{3}H]T_4-labelled DNA (800 000 cpm/μg).

as competitor is ~12% of the total radioactive RNA and ~3% when 30 min unlabelled RNA is used as competitor. This is almost the same as that obtained when the annealing is performed in the absence of DNA in an ordinary assay for symmetrical transcription. In the experiment with unlabelled early RNA this may effectively reduce some of the late sequences by complementarity instead of by competition.

Since RNA, RNA–DNA hybrids, double-stranded RNA and DNA have specific densities in Cs_2SO_4 [16], the products formed in the hybridization-competition experiment were analyzed by banding in the presence of this salt.

After completion of the hybridization reaction the samples were treated with S_1-nuclease in order to degrade all single-stranded nucleic acids remaining since these could interfere with the isopycnic banding of the different double-stranded products.

As seen from fig.3 the gradient analysis of the S_1-nuclease treated hybridization-competition mixture shows that at a high concentration with early or late RNA as competitor, the labelled RNA is found both in double-stranded RNA as well as in the hybrid. The two peaks in fig.3C show that some of the 20–30 min pulse-labelled RNA is present as hybrid and is not completely competed out by 30 min unlabelled RNA which should be expected from the nitrocellulose filter technique (fig.1) [1]. The hybrid peak in fig.3C may consist of hybrids which do not bind to the nitrocellulose filters because there may be some formation of perfect hybrids at the high concentration of unlabelled RNA used [14]. The relative amounts of radioactivity found in the hybrid and as double-stranded RNA using density gradients corresponds well with the data from the competition experiment in fig.2.

The density obtained for the nucleic acids in this experiment is somewhat higher than in [16] because the 0.1 M NaCl used in our gradients interferes with the density of the nucleic acids when they are dissolved in Cs_2SO_4.

4. Discussion

These data show that when large amounts of unlabelled T_4 RNA are used in the hybridization-competition experiment, complementary RNA present in the RNA populations forms double-stranded RNA in the annealing reaction. This double-stranded RNA is not detected when binding to nitrocellulose filters is used to assay the amount of radioactive hybrid formed.

Even at very high concentrations of competitor it is difficult to reach a plateau level when using the nitrocellulose filter assay. This may be because labelled RNA is removed as RNA–RNA duplexes. Furthermore, both the absence of a plateau level as wel as differences in the levels may vary because of variations between one RNA preparation and another in the ability to form such duplexes [7,17].

One problem in the evaluation of competition data with respect to RNA–RNA duplex formation is that only a fraction of the trichloroacetic acid-precipitable radioactivity anneals to DNA in the absence of unlabelled RNA and therefore is subject to competition. If the non-hybridized RNA is of the same composition as the hybridized RNA, duplex-formation should not considerably interfere with the results, but if the RNA which does not hybridize has a different composition, several implications could arise. Here, however, the results obtained by the nitrocellulose filter technique are in agreement with the data from the trichloroacetic acid-precipitation experiments when the amount of RNA–RNA duplex formed in fig.2B is subtracted from the curve in fig.2A.

Isopycnic centrifugation of the products after hybridization in the presence of high concentration of unlabelled RNA confirmed the results obtained with the trichloroacetic acid-precipitation analysis.

Although the formation of RNA–RNA duplexes in the specific hybridization experiment studied here does not change the main conclusions drawn from this experiment in [1], the fact that RNA–RNA duplex formation is not reduced by the presence of DNA may in other situations cause problems, e.g., in the analysis of RNA synthesized in vitro. Furthermore, since at a high concentration of unlabelled RNA some hybrids are not detected by the nitrocellulose filter method, the products should also be tested on isopycnic gradients.

When complementary RNA species are present as in the T_4 system, for example, conditions in the presence of formamide [18] or urea [19] should be worked out so that the formation of double-stranded RNA can be avoided (or at least reduced) so as to favour the formation of RNA–DNA hybrids.

Acknowledgements

This work was supported in part by grants from the Norwegian Research Council for Science and the Humanities, and from L. Meltzers Høyskolefond.

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