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A Method for the Hybridization of Nucleic Acid Molecules at Low Temperature*

James Bonner, Grace Kung, and Isaac Bekhor

ABSTRACT: Ribonucleic acid (RNA) may be hybridized to denatured deoxyribonucleic acid (DNA) at temperatures of 0–24° rather than the usual *ca.* 66°, provided that the hybridization reaction mixture contains an adequate concentration of formamide. The formamide concentration which yields maximum hybridization of RNA to DNA depends upon temperature and salt concentration. Maximum hybridization was achieved at 0° in a salt concentration of 0.16 M in 30 vol. % formamide, and at 24° in 0.32 M salt in the same formamide concentration. Under these two conditions the fraction of DNA hybridized with RNA at saturation is identical with the fraction of RNA hybridized at saturation under the usual hybridization conditions at elevated temperature. Advantages of the use of aqueous formamide over the use of high temperature include increased retention of DNA by membrane filters and lessened nonspecific absorption of RNA to such filters, as well as lessened chain scission of RNA during the long periods of incubation required for hybridization to saturation of complex RNAs to DNA.

We have found that RNA may be hybridized to denatured DNA at low temperature in the presence of appropriate concentrations of formamide. The procedure was originally developed during search for conditions under which RNA and protein might simultaneously be annealed to DNA. It has now been found, however, that the method has advantages over the use of heat for routine hybridization. We describe below the method and its merits.

The general procedures are based upon those of Gillespie and Spiegelman (1965) in which RNA in solution is hybridized to denatured DNA which is immobilized on nitrocellulose filters. Deproteinized pea DNA was further purified by treatment with RNase, pronase (preincubated to remove nuclease activity) to remove the RNase, and finally phenol extraction. It was then denatured in alkali and applied to 25-mm nitrocellulose filters (Schleicher & Schuell B-6) as described by Gillespie and Spiegelman (1965).

^{14}C-Labeled RNA was made by the transcription of pea cotyledon chromatin prepared according to the methods of Bonner *et al.* (1963). Such chromatin, which possesses a template activity 0.3 that of deproteinized pea DNA, was incubated under template-limiting conditions with purified *Escherichia coli* RNA polymerase (f_4 of Chamberlin and Berg, 1962) and ^{14}C-labeled ATP in the standard RNA polymerase reaction mixture of Chamberlin and Berg (1962). Incubation was for 2 hr at 30°. The RNA synthesized, which amounted to 40–90 times the amount of template used, was purified by phenol extraction, DNase (electrophoretically purified) treatment, and finally phenol extraction. The specific activity of the [^{14}C]ATP[1] used was adjusted so that the final RNA possessed a specific activity of approximately 75,000 cpm/OD_{260}. The RNA possessed an average sedimentation coefficient of 14 S in 0.1 M potassium acetate buffer.

In general, duplicate DNA-containing filters and one blank filter were incubated in 1 ml of RNA made up in salt of the required concentration and contained in a scintillation counting vial. Incubation was at various temperatures and for various periods of time detailed below. At the end of the incubation period the filters were removed, washed, treated with RNase, rewashed, and dried as recommended by Gillespie and Spiegelman (1962). The dried filters were then counted in a scintillation spectrometer.

When labeled RNA is incubated with DNA-con-

* From the Division of Biology, California Institute of Technology, Pasadena, California 91109. *Received August 11, 1967.* Report of work supported in part by U. S. Public Health Service Grant GM-13762.

[1] Abbreviations used: ATP, adenosine triphosphate; SSC, standard saline citrate.

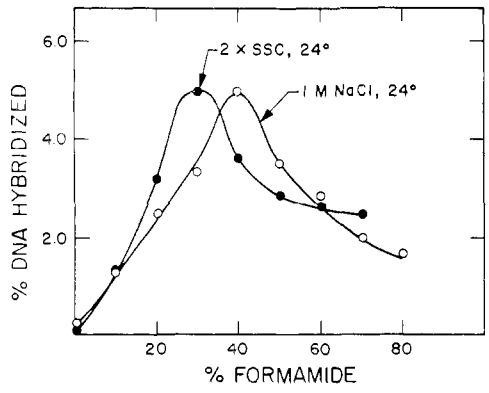

FIGURE 1: Hybridization at 24° of RNA transcribed from pea cotyledon chromatin to denatured pea DNA immobilized on nitrocellulose filters. Maximum hybridization is achieved at *ca.* 30% formamide in 2× SSC (0.32 M) and at *ca.* 40% formamide in 1 M NaCl. Hybridization for 20 hr. RNA concentration was 2.4 OD/ml (nonsaturating).

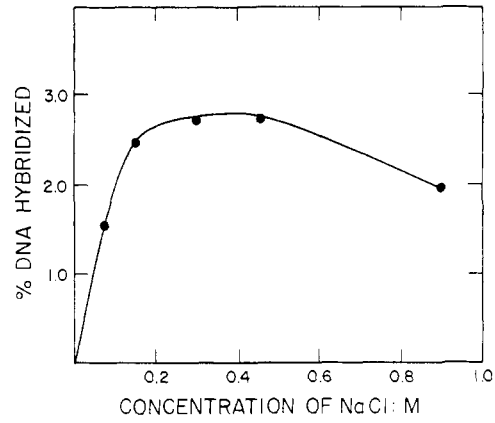

FIGURE 2: Hybridization at 24° of RNA transcribed from pea cotyledon chromatin to pea DNA immobilized on nitrocellulose filters. Maximum hydridization in 30% formamide is achieved at 0.3–0.45 M NaCl. RNA concentration was 1.5 OD/ml (nonsaturating).

taining filters in 2× standard saline citrate (SSC) at room temperature, no hybridization takes place over a period of 20 hr. If, however, the 2× SSC is made up to contain increasing amounts of formamide (99% purity, mp 2–3°, used without further purification), increasing amounts of hybridization take place, as is shown by the data of Figure 1. These data show that at 24° and in 2× SSC, the maximum amount of hybridization takes place at 30 vol. % formamide. The formamide concentration required for maximum hybridization is dependent on salt concentration.

The data of Figure 1 show, for example, that maximum hybridization takes place in 1 M NaCl in a formamide concentration of 40 vol. %, thus substantially greater than that required in 2× SSC. At a given formamide concentration, as 30%, maximum hybridization occurs over a broad range of salt concentrations (Figure 2). The data of Figure 3 show that hybridization of RNA to denatured DNA can be accomplished even at 0° provided that the ionic strength of the reaction mixture is sufficiently low (1× SSC) and the concentration of formamide sufficiently high (30 vol. %).

When RNA transcribed from pea cotyledon chromatin is hybridized to DNA in 2× SSC at 66° and at varying input RNA concentrations, saturation of the DNA with RNA is achieved when approximately 8% of the DNA is hybridized. Exactly the same value for saturation is achieved by hybridization in formamide at lower temperatures. The data of Figure 4 show that the fraction of DNA hybridized at saturation remains the same whether 66°–2× SSC, 24°–2× SSC–30% formamide, or 0°–1× SSC–30% formamide is used.

The final plateau level of hybridization (at input RNA concentrations of 6–10 OD_{260}) is achieved after approximately 18 hr for RNA of the complexity of that used in the present experiments. Rate of hybridization appears to be not greatly different between the three systems described in Figure 3.

Gillespie and Spiegelman report that denatured DNA immobilized on nitrocellulose filters according to their procedure remains quantitatively bound to the filter through incubation at elevated temperature and the subsequent washing procedures. We have found that while this is true of DNA immobilized on particu-

TABLE I: Retention of Applied Denatured Pea DNA to Varied Lots of Nitrocellulose Filters.[a]

Filter Lot	Condn of Incubn (°C)	Amt of DNA on Filter at Beginning of Incubn (OD_{260})	Amt of DNA on Filter after Incubn and Washing (OD_{260})
1	2× SSC (66)	1.0	0.72
2	2× SSC (66)	1.0	0.59
1	2× SSC (24) 30% formamide	1.0	1.06
2	30% formamide	1.0	0.91
1	1× SSC (0) 30% formamide	1.0	1.00
2	30% formamide	1.0	1.06

[a] Application of DNA to filters is described by Gillespie and Spiegelman (1965). Incubation for 20 hr in 2× SSC followed by washing of filters as described by Gillespie and Spiegelman. Amount of DNA on filter determined by hydrolysis (0.5 N perchloric acid, 10 min, 100°) and spectrophotometry.

TABLE II: Nonspecific Retention of Labeled RNA by Nitrocellulose Filters Lacking DNA under Different Conditions.[a]

Incubn Condn	RNA in Hybridization Mixture OD_{260}	RNA in Hybridization Mixture Cpm × 10^{-5}	RNA Hybridized (cpm)	Retention by Blank Filters Cpm	Retention by Blank Filters % of that Hybridized	Retention by Blank Filters % of Input
66°, 2× SSC	1.0	0.75	1,808	107	5.9	0.14
	3.0	2.25	4,080	339	9.8	0.18
	5.0	3.75	11,031	951	8.4	0.24
24°, 2× SSC 30% formamide	1.5	1.12	4,318	33	0.8	0.03
	3.0	2.25	7,462	131	1.8	0.06
	5.0	3.75	9,718	123	1.3	0.03

[a] Incubation for 20 hr followed by standard washing procedures.

lar lots of nitrocellulose filters, it is not true of all lots. The experiments of Table I summarize the behavior of lots of filters exhibiting poor retention of DNA. In two instances 28–41% of the DNA initially contained on the filter was lost during incubation at 66° and subsequent washing procedures. A virtue of the formamide hybridization method is that such loss of DNA is minimized, as is shown in Table I. The bonds which hold DNA to filter appear to be much less sensitive to formamide at room temperature than to 66°. Loss of DNA from filters in 30% formamide at 0° is negligible.

When a filter containing heterologous DNA, or no DNA at all, is immersed in a solution of labeled RNA and incubated at 66°, some labeled RNA, nonspecifically bound, remains associated with it through the subsequent washing and RNase procedures. This amount of nonspecifically bound RNA represents the background of the hybridization technique. The data of Table II show that the background is substantially reduced if formamide is substituted for 66°. Although the data of Table II concern nonspecific retention to filters containing no DNA, identical results were obtained with filters containing heterologous (rat) DNA.

Finally, when RNA is heated at 66° for extended periods of time it is extensively degraded. This is minimized by incubation at lower temperature in aqueous formamide. The data of Table III show, for example, that incubation in 2× SSC for 20 hr at 66°

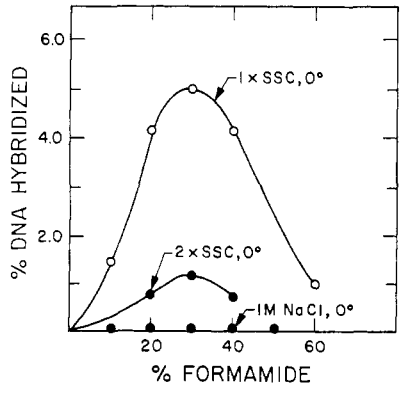

FIGURE 3: Hybridization at 0° of RNA transcribed from pea cotyledon chromatin to denatured pea DNA immobilized on nitrocellulose filters. Maximum hybridization is achieved at *ca.* 30% formamide in 1× SSC (0.16 M). At higher salt concentrations, no concentration of formamide suffices to permit of equally great hybridization at 0°. Hybridization was for 20 hr. RNA concentration was 2.4 OD/ml (nonsaturating).

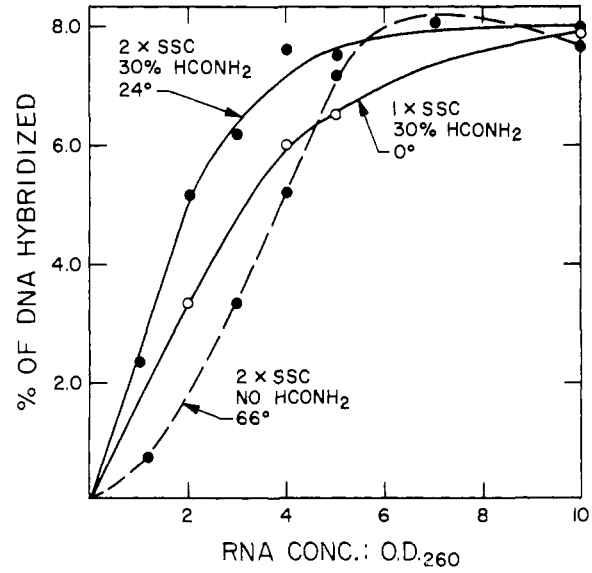

FIGURE 4: Fraction of denatured pea DNA hybridized at saturation by RNA transcribed from pea cotyledon chromatin under three different hybridization conditions: 66° for 22 hr in 2× SSC but without formamide, 2× SSC at 24° in 30% formamide, and 1× SSC at 0° in 30% formamide. The fraction of DNA hybridized at saturation is the same in all cases.

TABLE III: Sedimentation Coefficients of rRNA (Wheat) before and after 20-hr Incubation under Hybridization Conditions.

Condition of Incubation	$s_{20,w}$[a] (S)
Initial material	17.9, 25.0
66°, 2× SSC	2.61
24°, 2× SSC, 30 vol. %, formamide	17.0, 25.1
0°, 2× SSC, 30 vol. %, formamide	18.2, 24.8

[a] The RNA samples after incubation were dialyzed against water and sedimented (through D_2O containing 0.01 M Tris (pH 7.7) and 0.1 M NaCl) by the band sedimentation method of Vinograd *et al.* (1963). Data were recorded at 6-min intervals with the direct scanning system of the Spinco Model E centrifuge. We are indebted to Douglas Brutlag for his assistance.

degrades ribosomal RNA to an average sedimentation coefficient of 2.6. Incubation for a similar period in 2× SSC, 30 vol. % formamide at 24 or 0° causes no detectable change in the RNA.

That formamide should take the place of elevated temperature in the hybridization process is to be expected. Aqueous solutions of formamide denature native DNA as has been shown by Helmkamp and Ts'o (1961) and Marmur and Ts'o (1961). The concentrations of formamide required for DNA–RNA hybridization, 30–40 vol. %, are well below the 60 vol. % found by Marmur and Ts'o to be required for denaturation of native DNA (in 0.02 M NaCl–0.002 M sodium citrate).

What has now been found by serendipity is that hybridization as conducted in aqueous formamide solution possesses distinct advantages over hybridization conducted at elevated temperatures. These advantages include increased retention of immobilized DNA by the nitrocellulose filters and decreased nonspecific background absorption. These two factors combine to result in an increased reproducibility of replicates with the formamide procedure. Hybridization in formamide solution at low temperature is helpful also in minimizing chain scission of nucleic acid molecules during prolonged periods of incubation.

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