RNA-DNA HYBRIDIZATION IN SOLUTION WITHOUT DNA REANNEALING

Bert Vogelstein and David Gillespie

Section on Molecular Hybridization, Laboratory of
Tumor Cell Biology, National Cancer Institute
Bethesda, Maryland 20014

Received March 14,1977

SUMMARY: In solutions containing 70% formamide hybridization of RNA to denatured DNA proceeded in the absence of DNA reannealing at temperatures permitting the optimum rate of formation of RNA-DNA hybrids. At low complementary DNA to RNA ratios the percentage of E. coli ribosomal RNA hybridized was substantially higher in 70% formamide than in solutions lacking formamide. The ability to perform RNA-DNA hybridization without competing DNA reannealing facilitates the description of the kinetics and stoichiometry of the reaction.

INTRODUCTION

RNA-DNA hybridization has been used extensively in many areas of molecular biology. However, there are several technical problems that complicate the interpretation of results obtained with the technique. It has been shown, for example, that the rate of hybridization of RNA to DNA in aqueous solution is lower than the rate of the competing DNA reannealing reaction (1). For any given RNA sequence, complementary DNA must be in vast excess to achieve reasonably complete hybridization of the RNA. Moreover, DNA reassociation complicates formal treatment of the kinetics and stoichiometry of the RNA-DNA hybridization reaction. It has also been shown that optimum rates of formation of RNA-DNA hybrids in aqueous solution are only achieved at temperatures near 70°, tempatures leading to RNA instability. Hybridization reactions of extended duration are difficult to carry out at these temperatures.

Birnstiel et al. (2) showed that with immobilized DNA, RNA-DNA hybrids could be formed in 50% formamide at temperatures near the t_m of the resultant

ISSN 0006-291X

hybrid structures. Subsequently, White and Hogness (3) and Thomas et al. (4) developed a system using 70% formamide allowing RNA to replace a segment of one strand of locally denatured DNA, forming an "R-loop" structure. Thomas et al. (4) also showed that in 70% formamide the thermal stability of the RNA-DNA structure was greater than that of DNA duplexes of the same guanosine-cytosine content.

A condition where DNA duplexes are less stable than RNA-DNA hybrids offered a potential system for performing RNA-DNA hybridization with limited DNA reannealing, starting with single-stranded RNA and fully denatured DNA. The results of this communication show that in 70% formamide the optimum temperature for RNA-DNA hybridization is one where DNA reannealing is negligible and, additional is low enough to permit extended incubations without appreciable RNA degradation.

MATERIALS AND METHODS

DNA and RNA. E. coli DNA was obtained from Worthington Biochemicals and was sheared in a Britten-Davidson press at 50,000 psi to an average single-stranded size of 6-7S determined by sedimentation in alkaline sucrose. The DNA was further purified by extraction with phenol-chloroform, incubated for 20 hr. at 37° in 0.4 M NaOH, precipitated from ethanol, and dissolved in water or in formamide (Baker Chemicals, reagent grade). Formamide was stored at room temperature and not repurified before use but only pH 6-7.5 formamide was used. E. coli [^{3}H]DNA, a gift from D. Strayer, was sheared by sonication to a single-stranded size of 12S, then purified as described above. E. coli ribosomal RNA, labelled with [^{125}I] to a specific activity of $3.5 \cdot 10^{7}$ cpm/µg, was a generous gift from W. Prensky. This RNA was further purified by filtration through nitrocellulose membranes and chromatography in 50% ethanol-50% TNE (0.01 M tris-HCl, pH 7.5, 0.1 M NaCl; 0.001 M EDTA) on cellulose (CF11, Whatman). RNA was eluted from CF11 with TNE lacking ethanol and stored at -20°.

Molecular Hybridization in 70% Formamide. For a typical RNA-DNA hybridization experiment seven parts of DNA in 100% formamide were mixed with three parts of [^{125}I]RNA in 1.2 M potassium phosphate buffer pH 6.8. This solution is called formamide-phosphate. The final concentration of phosphate was 0.36 M, unless specified otherwise. Aliquots of 2 µl were flame-sealed in 5 µl capillary pipettes, incubated at 100° for 5 min., then incubated at hybridization temperature. Potassium phosphate was used because sodium phosphate precipitated from 70% formamide at high temperatures. At concentrations over 0.36 M, potassium phosphate also precipitated from 70% formamide. Slight crystallization did not interfere with the hybridization reaction. At 0.36 M and below, potassium phosphate remained dissolved in sealed capillaries, judged by refractometry. When higher [K^{+}] was desired, KCl was added to the hybridization mixture containing 0.18 M potassium phosphate.

After hybridization, the contents of the capillary tube were expelled into 750 µl of 0.45 M NaCl and 0.045 M Na citrate and divided into two aliquots. One aliquot was exposed to 20 µg/ml of RNase A (Sigma Chemicals) at 37° for 1 hr. The acid-precipitable radioactivity in this aliquot, relative to that in the

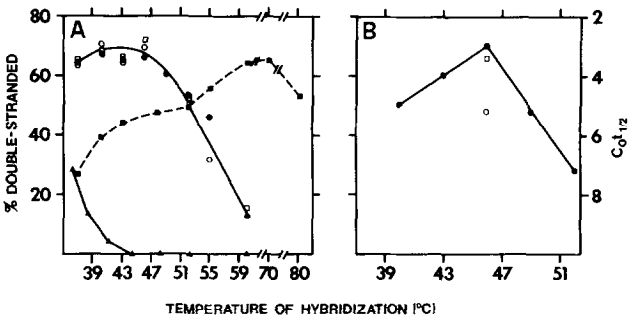

Figure 1A: Extent of DNA reannealing and RNA-DNA hybridization versus temperature and ionic strength. For DNA reannealing (▲), 14.0 µg of E. coli [^{3}H]DNA was sealed in 2 µl of 70% formamide containing 0.36 M potassium phosphate and denatured. Reannealing was carried out to a C_0t of 400 (4.7 hrs) and was monitored by chromatography on hydroxyapatite as described in Materials and Methods.

For RNA-DNA hybridization, 6.6 µg of E. coli DNA and 0.1 ng of E. coli [^{125}I] ribosomal RNA (3500 cpm) were sealed in 2 µl of formamide-phosphate or aqueous phosphate. Hybridization was carried out to a C_0t of 80 (2 hrs) and was monitored by RNAase resistance as described in Materials and Methods. ■ = 0.36 M aqueous phosphate, ○ = 70% formamide and 0.18 M potassium phosphate, final concentrations, ● = 70% formamide and 0.36 M potassium phosphate, final concentrations, □ = 70% formamide and 0.18 M potassium phosphate and 0.68 M potassium chloride, final concentrations.

Figure 1B: Rate of RNA-DNA hybridization in 70% formamide versus temperature and ionic strength. E. coli DNA (1 µg) and E. coli [^{125}I] ribosomal RNA (0.1 ng, 3500 cpm) were sealed in 2 µl of formamide phosphate and the kinetics of formation of RNAase-resistant hybrids were followed as described in Materials and Methods. The rate, as defined by the C_0t required to reach half the maximum achievable value (see Figure 1A) was determined for each kinetics curve and plotted on the abscissa. The C_0t value (DNA concentration times time) is taken from the standard condition of Britten and Kohne (6), without correction for temperature or ionic strenth. Symbols are the same as in Figure 1A.

aliquot not treated with RNAse, was used to calculate the percent of the RNA hybridized (5). Acid-precipitability obtained at zero time was less than 2% of the input values.

Molecular Hybridization in 0.36 M Potassium Phosphate. This was carried out as described above, except that DNA was dissolved in water instead of formamide.

DNA Reannealing. Seven parts of E. coli [^{3}H]DNA, dissolved in 100% formamide was mixed with three parts of 1.2 M potassium phosphate, then aliquoted, denatured, and incubated under hybridization conditions as described above. After reannealing, the mixture was diluted with 0.14 M sodium phosphate, pH 6.8 and reannealed DNA was bound to hydroxyapatite. Residual unannealed DNA was washed from the hydroxyapatite and the hydroxyapatite was collected by centrifugation. Reannealed DNA was estimated by dissolving the hydroxyapatite in 10% trichloroacetic acid and measuring acid-precipitable radioactivity.

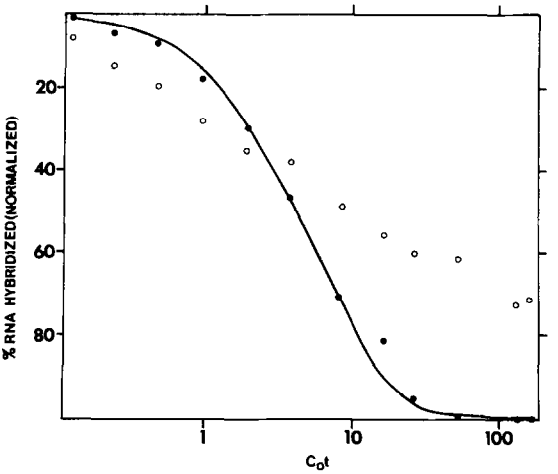

Figure 2: Kinetics of RNA-DNA hybridization at modest DNA excess. E. coli DNA (190 ng) and E. coli [^{125}I] ribosomal RNA (0.1 ng, 3500 cpm) were sealed in 2 µl formamide-phosphate or aqueous phosphate, using 0.36 M phosphate in both cases. The ratio of rDNA to rRNA = 5.7 to 1.0, assuming that 0.3% of the DNA is complementary to ribosomal RNA (7-9). The kinetics of formation of RNAase-resistant hybrids were followed as described in Materials and Methods. The C_0t values plotted on the abscissa are uncorrected for temperature and ionic strength. A C_0t of 1 corresponds to 0.86 hrs of hybridization. The percent hybridization values are normalized to account for RNA failing to hybridize at high C_0t (see Figure 1A) after subtracting RNAase-resistance at zero time (<2% of input radioactivity). The solid line represents the theoretical curve expected for a simple second-order reaction where one of the components is in modest excess. ● = formamide-phosphate; ○ = aqueous phosphate.

RESULTS AND DISCUSSION

Figure 1 shows that while reannealing of E. coli DNA was prevented at temperatures above 41° in 70% formamide buffered with potassium phosphate, the "final" extents (Figure 1A) and rates (Figure 1B) of hybridization of ribosomal RNA to DNA were optimal, within a factor of two, between 40 and 50° and between 0.3 M and 0.9 M cation concentration. The temperature optimum in aqueous phosphate buffer was around 70°.

The kinetics of RNA-DNA hybridization in formamide phosphate is shown in Figure 2. The experimental points (closed circles) were in close agreement with a theoretical curve expected of a simple second-order reaction where one of the

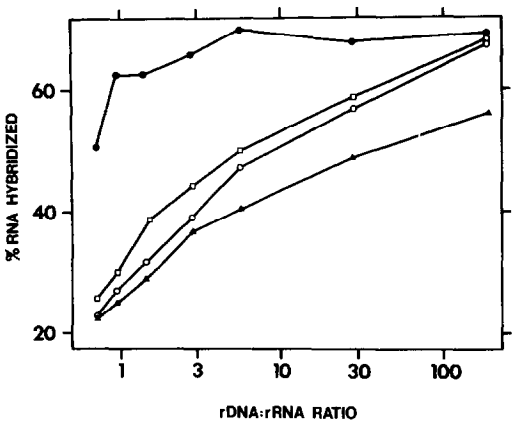

Figure 3: Extent of RNA-DNA hybridization versus the concentration of complementary DNA relative to RNA. Appropriate amounts of E. coli DNA and E. coli [^{125}I] ribosomal RNA were sealed in 2 µl of formamide phosphate or aqueous phosphate. Hybridization was carried out to a C_0t of 100, uncorrected for ionic strength but corrected for temperature in the cases of the 70° and 80° incubations. At complementary DNA:RNA ratios of 5.6 and above, 0.1 ng of RNA was mixed with 0.19-6.6 µg of DNA; at DNA:RNA ratios of 2.8 and below, 0.2-0.8 ng of RNA was mixed with 0.19 µg of DNA. The ratio of complementary ribosomal DNA to ribosomal RNA, plotted on the abscissa, was determined assuming that 0.3% of the total DNA is complementary to ribosomal RNA (7-9). These values are only approximate because of the uncertainty in copy number of the ribosomal genes and the difficulty in determining the exact specific activity of the [^{125}I] RNA. ● = formamide phosphate, 46°; □ = aqueous phosphate, 70°; ○ = aqueous phosphate, 60°; ▲ = aqueous phosphate, 80°. Potassium phosphate at 0.36 M final concentration was used throughout.

reactants is in modest excess (solid line). In comparison, the kinetics in aqueous phosphate was not as easily interpretable. The rate of RNA-DNA hybridization, defined as the time needed to reach half-maximal RNA hybridization for each condition, was two times slower in formamide-phosphate at 46° than in aqueous phosphate at 60° (figure 2 and ref. 1). There was no detectable RNA degradation for several days at 46° in formamide-phosphate, measured by loss of acid-precipitable radioactivity. Maximally, seventy percent of the RNA could be driven into RNAase-resistant structures, with or without formamide present.

The complex kinetics of RNA-DNA hybridization in aqueous phosphate at low ratios of complementary DNA to RNA (figure 2) could result from a competing reaction involving DNA reannealing. As shown in figure 3, the DNA to RNA ratio

greatly affected the extent of RNA hybridization in the absence of formamide; vast DNA excesses were required to drive the reaction toward completion at all temperatures of hybridization tested. In contrast, RNA-DNA hybridization proceeded at high efficiency in 70% formamide, even at complementary DNA to RNA ratios approaching unity.

These experiments show that in 70% formamide buffered with phosphate RNA-DNA hybridization can be carried out without significant DNA reannealing. As a result it is now possible to treat the kinetics and stoichiometry of this reaction formally, calculating important parameters such as number of copies of complementary DNA and predicting with some certainty the amount of DNA that will be required to drive any given RNA species quantitatively into hybrid structures. Since DNA is soluble in formamide at over 100 mg/ml (unpublished observations) the minor problem of a slightly slower reaction in solutions containing formamide can be overcome by using higher DNA concentrations. Additionally, the relatively lower temperature of hybridization in 70% formamide permits extended incubations.

After this work was completed we learned that Casey and Davidson (personal communication) have also studied RNA-DNA hybridization in solutions containing high percentages of formamide with results similar to ours.

ACKNOWLEDGEMENTS

We thank R.C. Gallo for advice and support. We thank W. Prensky and D. Strayer for gifts of [^{125}I]RNA and [^{3}H]DNA, respectively.

REFERENCES

1. Melli, M., Whitfield, C., Rao, K. V., Richardson, M. and Bishop, J. O. (1970) Nature New Biol. 231, 8-12.
2. Birnstiel, M. L., Sells, B. H., and Purdom, I. F. (1972) J. Mol. Biol. 63, 21-39.
3. White, R. L. and Hogness, D. S. (1977) Cell, in press.
4. Thomas, M., White, R. L., and Davis, R. W. (1976) Proc. Nat. Acad. Sci., U.S.A., 73, 2294-2298.
5. Gillespie, D., Gillespie, S., and Wong-Staal, F. (1975) Meth. Canc. Res. 11, 205-245.
6. Britten, R. J., and Kohne, D. E. (1968) Science 161, 529-540.
7. Gillespie, D. and Spiegelman, S. (1965) J. Mol. Biol. 12, 829-842.
8. Loening, U. E. (1967) Biochem. J. 102, 251-257.
9. Steele, W. J. (1968) J. Biol. Chem. 243, 333-341.