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RNA:DNA hybrids are more stable than DNA:DNA duplexes in concentrated perchlorate and trichloroacetate solutions

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ABSTRACT

Rates of formation of RNA:DNA hybrids have been measured as a function of temperature and compared to DNA:DNA duplex denaturation temperatures in 4 M sodium perchlorate, 4 M $NaClO_4$-6 M urea, and 3 M rubidium trichloracetate solvents. The usual bell shaped curves of reaction rate versus temperature were observed. The optimal temperatures for the RNA:DNA association reaction are 5° to 12° greater than the T_m's for DNA:DNA denaturation in these solvents, just as in formamide. R-loops of $\phi 80 d_3 ilv$ DNA with E. coli rRNA can be formed at high efficiency in these solvents.

INTRODUCTION

High concentrations of certain electrolytes, including sodium perchlorate and trichloroacetates (1), as well as various neutral molecules, including formamide (2), dimethyl sulfoxide (3), and urea (4), lower the melting temperature of duplex DNA and therefore the optimum temperature for DNA:DNA reassociation (5,6). This is advantageous because, in general as the reaction temperature is lowered, the amount of chain scission occurring during the time needed for association reactions of complementary strands is reduced.

Aqueous formamide solvent systems are particularly useful for preparing RNA:DNA hybrids, because the hybrids are more stable than DNA:DNA duplexes in these solvents. It is therefore possible to find conditions under which incubation of RNA with DNA duplexes leads to the formation of R-loops (7) and conditions under which RNA:DNA hybrids will form from the respective single strands but DNA:DNA duplexes are unstable and do not form (8). These conditions are useful for a number of applications (9,10).

It is of interest to determine whether other denaturing solvents have the property of favoring the formation of RNA:DNA hybrids over that of DNA:DNA duplexes. We report here a preliminary survey of aqueous $NaClO_4$, $NaClO_4$-urea, and Rb Cl_3CCO_2 as solvents for RNA:DNA association reactions.

MATERIALS AND METHODS

Materials. Reagent grade disodium ethylene diaminetetraacetic acid (EDTA) and sodium acetate (NaAc) were from J.T. Baker; urea, Ultra Pure, from Schwartz-Mann; and $NaClO_4$ from the G. Frederick Smith Chemical Company. Rubidium trichloracetate (RbTCA) was a gift from R.L. Burke and W.R. Bauer (11).

E. coli 23S rRNA was labeled with ^3H to a specific activity of 2.2×10^5 cpm/μg and extracted according to published procedures (12). The molecular length of this RNA was measured to be 3.2 kb by agarose gel electrophoresis in the presence of methylmercuric hydroxide (13). E. coli DNA was purchased from the Sigma Company, subjected to two phenol extractions and then sedimented on an alkaline sucrose gradient (0.1 M NaOH, 0.9 M NaCl, 5-20% sucrose) with linear SV40 single stranded DNA as a marker. Only those fractions sedimenting faster than the marker (i.e., greater than 5 kb in length) were collected and used. The upper limit of length of the DNA strands in the preparation was about 8 kb. $\phi 80 d_3 ilv$ phage were grown and DNA prepared as previously described (14).

Methods. Thermal denaturation curves of E. coli DNA were determined by absorbance measurements at 270 nm with a Gilford Model 2000 recording spectrophotometer fitted with a linear temperature programmer. The heating rate was 0.5°C/min.

RNA-DNA hybridization measurements were carried out at concentrations of ^3H-labeled E. coli 23S rRNA of 1 μg/ml and E. coli DNA of 12 μg/ml; that is with an approximately 32 fold RNA sequence excess. The reactions were carried out in the following solvent systems: 1) 4 M $NaClO_4$, 0.01 M EDTA, pH 7.0; 2) 4 M $NaClO_4$, 0.01 M EDTA, 6 M urea, pH 7.0; 3) 3 M RbTCA, 0.03 M Tris, 0.003 M EDTA, pH 7.5; 4) 2 M NaAc, 0.02 M EDTA, pH 6.3. Aliquots were withdrawn from the incubation mixture at various times, diluted 10 fold with 1.5 x SSC (SSC is 0.15 M NaCl, 0.015 M Na_3 citrate, pH 7.75) and incubated at 37°C for 2 hours in the presence of 40 μg/ml of RNAase A and 1.3×10^3 units/ml RNAase T_1. Samples were then precipitated with 8% TCA, collected on glass filters (Whatman GFC) and assayed for radioactivity.

R-loops were formed by incubation of 8μg/ml of E. coli 23S rRNA and 8 μg/ml of $\phi 80 d_3 ilv$ DNA (thus representing approximately a 33 fold RNA sequence excess) in the 4 M $NaClO_4$, 4 M $NaClO_4$-6 M urea and 3 M RbTCA solvents described above. For 4 M $NaClO_4$, the first incubation step was at 90° for 1 min, then 67.5° for 30 min, then 40°C for 30 min; for the 4 M $NaClO_4$-6 M urea solvent, incubation was at 52°C for 30 min, then at 15°C for

30 min; with 3 M RbTCA, the first 30 min. incubation was at 56°, and the second at 20°C. Solutions were quenched to 4°C and stored, diluted 30 fold into 55% formamide, 0.06 M Tris, 0.006 M, EDTA, pH 8.5, 0.06 mg/ml cytochrome-c for spreading onto a 17% formamide, 0.01 M Tris, 0.001 M EDTA, pH 8.5 hypophase. Samples were mounted for electron microscopy by standard methods. Circular double stranded SV40 DNA was used as an internal length standard, with a length taken as 5.38 kb.

RESULTS

RNA:DNA Association Rates. The extent of reaction between E. coli 23S rRNA present in about a 32 fold molar excess and denatured E. coli DNA was measured as a function of time at several temperatures in several solvent systems by assaying for the amount of ribonuclease resistant RNA as described in Materials and Methods. As shown in Fig. 1, time points were taken for a series of temperatures up to about that corresponding to the maximum rate. Only a single time point at a rot value of 2.36×10^{-3} M sec was measured at higher temperatures; these data are presented in Fig. 2. This time point corresponds to an intermediate degree of reaction at all temperatures. The amount of reaction indicates the relative magnitude of the reaction rate as a function of temperature, although quantitative rate constants cannot be calculated from the single data points at the high temperatures. We designate the temperature at the maximum of the curves as the optimum temperature, T_{optRD}, for RNA:DNA hybridization.

The results with $NaClO_4$ (Fig. 1) show the following features: a) The plateau value for the extent of reaction increases with increasing temperature from 40° to 70°. b) The $rot_{\frac{1}{2}}$ (defined relative to the plateau) decreases with increasing temperature (7×10^{-3}, 1.5×10^{-3}, 1.2×10^{-3} and 8.5×10^{-4} M sec at 40°, 50°, 60°, and 70° respectively). We shall suggest in the DISCUSSION section that a more reasonable comparison of rates at different temperatures is based on a comparison of initial slopes, -d(cpm)/dt. For a rough approximation of relative initial slopes we take the times required for 200 cpm to become RNAase resistant, and estimate relative rates of 1, 0.45, 0.34, and 0.05 at 70°, 60°, 50°, and 40°, respectively. c) The temperature dependence of the total amount of reaction for rot = 2.36×10^{-3} M sec is bell shaped (Fig. 2) with T_{optRD} = 70°C. This temperature is 5° higher than the T_m of E. coli DNA measured in this solvent (table 1) and thus about 25° greater than the optimal temperature for DNA:DNA reassociation (5,6).

A clear cut plateau was not reached at 35° for $NaClO_4$-urea, but the reaction rate clearly increased from 35° to 45° to 53°. Again, the optimum

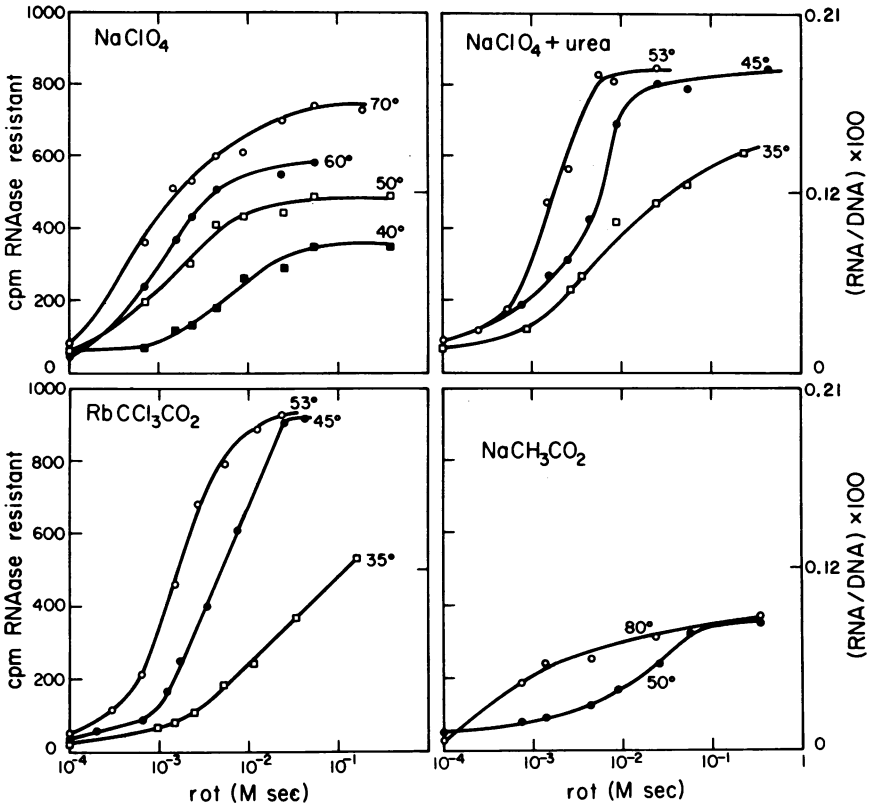

Fig. 1. Reaction of excess E. coli 23S rRNA with total E. coli DNA as a function of time and temperature in several electrolyte systems.

temperature for hybridization, as judged by incubation to a rot of 2.36 x 10^{-3} M sec (Fig. 2b), was 52°-53°, which is about 12° above the melting point of DNA:DNA duplexes. The results in Rb Cl_3CCO_2 are rather similar to those in $NaClO_4$-urea; the optimal temperature for RNA:DNA hybridization was 56°, and the T_m of E. coli DNA, 45°C.

As an example of a concentrated non-denaturing salt, we chose 2 M sodium acetate, pH 6.3. Measurements in this solvent are also presented in Figs. 1 and 2. In this case, the optimum temperature, T_{optRD}, is less than the T_m of DNA.

The E. coli genome contains "at least seven" rRNA gene sets (15). Seven 23S rRNA genes are 0.26% by weight of the E. coli genome. The observed

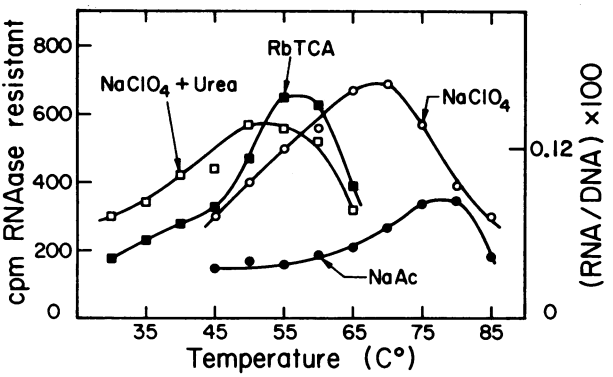

Fig. 2. Extend of reaction between excess 23S rRNA and DNA as a function of temperature at rot = 2.36×10^{-3} M.

maximum levels of hybridization in Fig. 1 are 83%, 76%, 66%, and 31% of this value for Rb CCl_3CO_2, $NaClO_4$-urea, $NaClO_4$, and $NaCH_3CO_2$ respectively. Thus, a larger degree of reaction is achieved in the denaturing solvents than in $NaCH_3CO_2$.

The data comparing T_{optRD} with the T_m for E. coli DNA are summarized in Table 1.

Table 1.

Comparison of optimum temperature for 23S rRNA:DNA hybridization with melting temperature* of E. coli DNA.

Salt	4 M $NaClO_4$	4 M $NaClO_4$ 6 M urea	3 M RbTCA	2 M NaAc
T_{optRD}	70°C	52°C	56°C	80°C
T_m	65°C	40°C	45°C	95-100°C**
$T_{optRD}-T_m$	5°C	12°C	11°C	-10°C

*T_m defined as the midpoint of the thermal denaturation curve measured by A_{270}

** estimated from the data of Schildkraut and Lifson (16).

R-loop formation. The data presented so far show that RNA:DNA hybrids are more stable than DNA:DNA duplexes in perchlorate and trichloroacetate solvents. Therefore, it should be possible in such solvents to form R-loops which can be observed by electron microscopy. We have used the technique introduced by Holmes et al. (17) to study R-loop formation in the solvents

of interest here. This technique, which is applicable to simple genomes, invloves complete strand dissociation of the DNA, incubation with RNA coded for by a segment of the DNA at a temperature at which RNA:DNA hybrids will form but at which DNA:DNA duplexes will not (and may, in fact, be unstable) followed by further incubation at a lower temperature to achieve DNA:DNA reassociation. The test system used is the DNA of $\phi 80 d_3 ilv$ which contains one E. coli 23S rRNA gene (14, 18).

Detailed conditions for the formation of the R-loop molecules are given in Materials and Methods. We find that under these conditions, 65-75% of the $\phi 80 d_3 ilv$ molecules have R-loops (Table 2). An electron micrograph of a typical molecule is shown in Fig. 3. The lengths of the RNA:DNA hybrid region and the coordinates of the 23S rRNA gene are shown in Table 3.

Chain scission and RNA solubility.

For various kinds of RNA:DNA hybridization studies, the important practical requirements are that there are association conditions which allow the selective preparation of RNA:DNA hybrids without formation of DNA:DNA duplexes, without too much RNA or DNA chain scission, and with adequate nucleic acid solubility. Experiments were performed to estimate the amount of scission of the DNA molecules under conditions of typical hybridization incubations. ^3H labeled 23S rRNA solutions were incubated for varying times and temperatures and subjected to agarose-CH_3HgOH gel electrophoresis. Fractions were cut from the gels and counted. An overall summary of the results is as follows (data not shown). In 4 M $NaClO_4$, about 50%

Table 2

Efficiency of R-loop Formation*

Solvent	Double-stranded molecules with an R-loop	Double-stranded molecules without an R-loop	Molecules which are partially duplex, partially single-stranded	Single-stranded molecules
4 M $NaClO_4$	61	15	21	4
4 M $NaClO_4$-6 M urea	51	24	17	8
3 M RbTCA	55	13	24	8

*R-loops were formed as described in the text. Random fields were photographed and molecules counted. For those molecules which were partially duplex and partially single-stranded, it is not possible to score whether or not they could have formed R-loops.

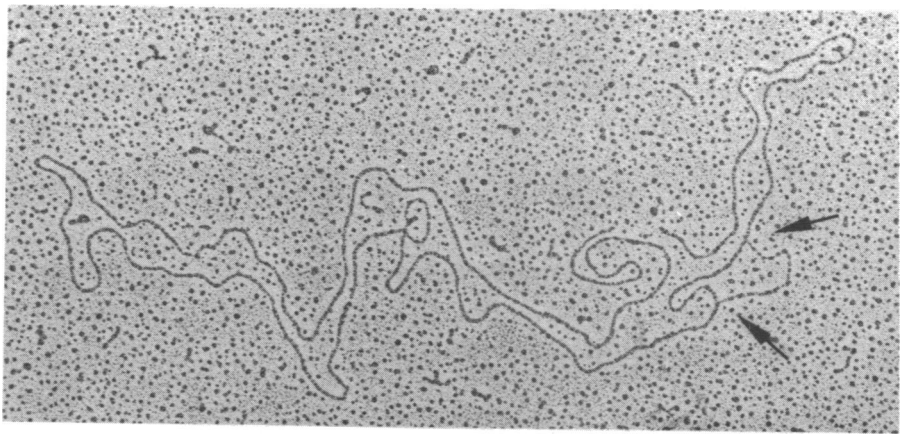

Fig. 3. Electron micrograph of $\phi 80 d_3 ilv$ duplex DNA with an R-loop (marked by arrows) of 23S rRNA. For this molecule, the coordinates of the R-loop are 8.31 and 10.51 kb, with a length of 2.20 kb.

of the molecules underwent one or more breaks in 60 min. at 56° but there was very little breakage at 50°C. In 3 M Rb Cl_3CCO_2, there was 50-75% breakage in 60 min. at 56°. In 4 M $NaClO_4$-6 M urea, there was 50% breakage in 30 min. at 50°C and all of the molecules were cleaved at least once by incubation at 60 min. at 50°C. We believe that by proper control of pH and by removal of heavy metal ions, the amount of RNA chain scission observed

Table 3

Coordinates of R-loops for $\phi 80 d_3 ilv$:23S rRNA*

Solvent	Average length of R-loop	Coordinates of left and right ends of R-loops	
4 M $NaClO_4$	2.35 ± 0.20 kb	7.92 ± 0.29 kb	10.37 ± 0.20 kb
4 M $NaClO_4$-6 M Urea	2.35 ± 0.20 kb	7.92 ± 0.29 kb	10.27 ± 0.30 kb
3 M RbTCA	2.35 ± 0.20 kb	7.92 ± 0.29 kb	10.17 ± 0.20 kb

*Coordinates of the R-loops for all the molecules in Table 1 were measured relative to the defined (14) left end of the molecule. Ohtsubo et al (14) measured a length of 3.2 kb (7.3-10.5 kb) for the 23S rRNA gene hybridized to a single strand of $\phi 80 d_3 ilv$ (in a $\phi 80 d_3 ilv/\phi 80$ heteroduplex). Wu and Davidson (18) measured a length and coordinates of 2.9 kb (7.6-10.5 kb) on hybrids of 23S rRNA with single strands of $\phi 80 d_3 ilv$ and spread by the gene 32-Etd Br technique. Thus, the R-loops observed here are about 0.77 of the lengths observed by the above methods. This is probably due to displacement of part of the RNA:DNA duplex by branch migration.

by us can be reduced. However while the present experiments were in progress, it became apparent that, with the pH 6.4-6.8 PIPES buffer system developed by P. Chandler (8) to reduce the amount of RNA chain scission, formamide solvents are satisfactory for present applications (9,10; Y. Chien, personal communication).

The solubility of 23S rRNA in 4 M $NaClO_4$ 3 M RbTCA and 4 M $NaClO_4$ 6 M urea is about 140 µg/ml for each case. 7 M $NaClO_4$ lowers the T_m of DNA to about 20° below that of 4 M $NaClO_4$. This would seem to be advantageous for reducing chain scission. However, RNA is quite insoluble in 7 M $NaClO_4$ (E.P. Geidushek, personal communication).

DISCUSSION

Our main observation is that, in concentrated sodium perchlorate, concentrated rubidium trichloroacetate and sodium perchlorate-urea solutions RNA:DNA hybrids are more stable than DNA:DNA duplexes and that conditions may be selected in which RNA:DNA association occurs with no significant amount of DNA:DNA reassociation. The difference in stability between RNA:DNA hybrids and DNA:DNA duplexes in the usual aqueous solutions of non-denaturing salts (8, 19, 16) is much smaller than the differences observed for the above mentioned electrolytes and for formamide (8).

Concentrated $NaClO_4$, $RbCCl_3CO_2$, $NaClO_4$-urea as well as formamide solutions are all denaturing solvents for DNA. It has been proposed that these solvents denature because they stabilize unstacked purine and pyrimidine rings immersed in water by virtue of a "chaotropic" effect on the structure of water (1). We propose that in general DNA single strands are stabilized more than RNA single strands relative to duplex polynucleotides in solvents such as these in which the strength of hydrophobic interactions is decreased. Therefore, under some conditions the DNA:DNA hybrids may already dissociate into single strands, but the RNA:DNA hybrids remain as duplex. It may be surmised that other denaturing chaotropic electrolyte systems, for example with trifluoroacetate or thiocyanate anions (1), will have similar properties.

The relative effects of these "chaotropic" solvents on DNA:DNA reassociation rates as compared to RNA:DNA association rates may be analyzed by comparing the DNA:DNA and RNA:DNA association rates in 4 M $NaClO_4$ to those in aqueous NaCl solutions and in formamide. In 4 M $NaClO_4$ at 70°C (the T_{opt} for RNA:DNA association in this system) the observed $\underline{rot}_{\frac{1}{2}}$ is 8.5×10^{-4} M sec, corresponding to a rate k_{RD} for RNA:DNA association of 810 M^{-1} sec^{-1}. Chang et al. (6) report that the maximum rate constant k_{DD} for DNA:DNA re-

association in 4 M $NaClO_4$ (at 42°C for T4 DNA) is about 2/3 of that for 0.4 M NaCl. The rate constant in 0.4 M NaCl is about 0.45 times that in 1.0 M NaCl (20). Thus from the equation $k_{DD} = 3 \times 10^5 L^{0.5}/N$ for aqueous 1 M NaCl (5) and the above ratios, we calculate for L = N = 3200, $k_{DD} = 1.8 \times 10^3$ M^{-1} sec^{-1} in 4 M $NaClO_4$. So $k_{RD}/k_{DD} = 0.45$ in 4 M $NaClO_4$. For full length E. coli 23S rRNA reacting with long E. coli DNA in 80% formamide, 0.4 M NaCl, $k_{RD} = 230$ M^{-1} sec^{-1} at 45°C (P. Chandler, personal communication). For DNA strands with L = N = 3200, i.e. the length and complexity of 23S rRNA, the above equation gives $k_{DD} = 2.4 \times 10^3$ M^{-1} sec^{-1} in 0.4 M NaCl solution. It has been reported that the DNA:DNA reassociation rate constant in 80% formamide, 0.4 M NaCl is about 1/3 to 1/3.6 of that in aqueous solution (8). Therefore $k_{RD}/k_{DD} \approx 0.3$ in this solvent. Thus, in the relative effects on DNA:DNA as compared to RNA:DNA rate constants, the denaturing anions do not differ greatly from formamide.

The RNA:DNA association rate decreases with decreasing temperature. For example, the relative rates as calculated above in 4 M $NaClO_4$ are 1, 0.45, 0.34 and 0.05 at 70°C, 60°C, 50°C and 40°C respectively. This is an example of the characteristic decrease in reaction rate with decreasing temperature below the optimum (5). We presume that since RNA:DNA base pairs are more stable than DNA:DNA base pairs that RNA:RNA base pairs are even more stable (8, 21). The rate decrease is therefore attributed to a decrease in the RNA:DNA nucleation rate because of the increase in RNA secondary structure as the temperature decreases.

There is a very clear decrease in the apparent limiting value of the degree of reaction in 4 M $NaClO_4$ as the temperature decreases from 70°C to 40°C. We believe that several factors may contribute to this phenomena. The most important one is due to the great decrease in the RNA:DNA rate from 70°C to 40°C. 40°C is 25°C below the melting temperature of DNA, therefore, it is close to the optimum temperature for DNA:DNA reassociation. As discussed above, the RNA:DNA association rate constant is then 0.023 (0.45 x 0.05) of that of DNA:DNA reassociation. Despite the 30 fold sequence excess of RNA over complementary DNA the DNA:DNA reassociation rate becomes comparable to that of RNA:DNA reassociation. Therefore, the DNA:DNA reassociation removes sequences before they can react with RNA. At 40°C, the plateau level of RNA:DNA hybridization is reached at rot $\approx$ 0.03 M sec (Fig. 1). The corresponding cot value is 0.47 M sec. We calculate from the DNA:DNA rate constant given above that the seven fold reiterated rDNA sequences would have decrease in concentration to 0.45 of its initial value due to DNA:DNA

reaction alone at this time. The above interpretation predicts that at a higher ratio of RNA to DNA, more RNA:DNA reaction would be observed. These experiments are technically difficult because of the small fractional reaction of the RNA. A more promising approach would be to study the degree of reaction in a system with strand seperated DNA. An additional factor may contribute to the decrease in RNA:DNA reaction. The mechanism is based on the following hypotheses: 1) RNA:RNA base pairs are more stable than RNA:DNA duplexes in these solvents; 2) at the lower temperature RNA:DNA hybridizations in formamide or $NaClO_4$, RNA:DNA nucleation occurs and some RNA:DNA base pairs are formed but some of the RNA:RNA secondary structure features do not branch migrate out to give RNA:DNA base pairs; 3) the RNA secondary structure features are digested in the RNAase assay. It is conceivable that the formation of underwound loops between complementary RNA:RNA and DNA:DNA secondary structure features, as discussed by Broker et al. (22) stabilizes the secondary structure features, and prevents branch migration.

We conclude by reemphasizing that the important result of our study is that RNA:DNA hybrid formation is favored over DNA:DNA duplex formation in concentrated $NaClO_4$ and Rb CCl_3CO_2 salt solutions at suitable temperatures, just as in formamide solvents.

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