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Studies on nucleic acid reassociation kinetics: Retarded rate of hybridization of RNA with excess DNA*

(plasmid RSF2124 nucleic acids/bacteriophage φX174 nucleic acids/*Escherichia coli* nucleic acids)

GLENN A. GALAU†, MICHAEL J. SMITH†‡, ROY J. BRITTEN§¶, AND ERIC H. DAVIDSON†

† Division of Biology, California Institute of Technology, Pasadena, California 91125; and § Kerckhoff Marine Laboratory, Division of Biology, California Institute of Technology, Corona del Mar, California 92625

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ABSTRACT **The rate of reaction of excess double-stranded bacteriophage φX174 and plasmid RSF2124 DNA drivers with enzymatically synthesized asymmetric RNA tracers was measured. Other reactions were carried out with excess *Escherichia coli* DNA and *E. coli* RNA labeled *in vivo*. RNA and DNA fragment lengths were held approximately equal. For each case it was shown that in DNA excess the rate constant for RNA·DNA hybridization is 3- to 4.5-fold lower than that of the renaturation rate constant for the driver DNA. This retardation was also observed in pseudo-first-order hybridization reactions driven by excess strand-separated RSF2124 DNA. It was concluded that the rate constant for RNA·DNA hybridization depends partially on which species is in excess.**

Studies of RNA·DNA hybridization kinetics carried out in RNA excess (1) and in sequence equivalence (2) have shown that the rate constant for hybridization differs by less than 20% from the DNA renaturation rate constant. However, other reports in the literature indicate that in conditions of DNA excess the rate of hybridization is several-fold lower than the rate of renaturation of the driver DNA in the same reaction mixture (e.g., refs. 3 and 4). These seemingly contradictory data suggest a dependence on DNA/RNA ratio that is not expected for a simple collision-dependent process. The present experiments were undertaken to explore this effect in well-defined experimental systems. We find that relative retardation of DNA·RNA hybridization can be demonstrated convincingly with phage and plasmid nucleic acids, and in this communication report some of the circumstances under which this result can be obtained.

MATERIALS AND METHODS

Preparation of Nucleic Acids. The preparation, shearing, and sizing of *Escherichia coli* DNA, bacteriophage φX174 DNA, and φX174 (+)strand RNA have been described previously (1, 4, 5). Details of the preparation and shearing of sea urchin (*Strongylocentrotus purpuratus*) DNA and oocyte RNA (rRNA) have also been published (1, 6, 7).

E. coli [^{3}H]RNA synthesized *in vivo* was prepared essentially according to ref. 8. After extensive purification, the total RNA was fractionated by centrifugation in aqueous sucrose gradients. RNA averaging 400 nucleotides (NT) was recovered and used in these experiments.

Plasmid RSF2124 DNA (11,000 NT) was labeled with [^{3}H]thymine during chloramphenicol-induced amplification in *E. coli* strain C600 (9). Supercoil DNA was isolated by Brij lysis followed by buoyant density centrifugation in ethidium bromide/CsCl. Radioactive DNA was linearized with *Bam* I (BioLab) and unlabeled DNA, with *Eco*RI (Miles Laboratories) restriction endonucleases. Strands were separated by CsCl buoyant density centrifugation, following denaturation and reaction with poly(U,G) (Miles Laboratories). The preparations were renatured and the duplex fraction was removed by hydroxyapatite (HAP) chromatography. Supercoils or strand-separated DNAs were sheared by sonication. The [^{3}H]RNA·DNA was synthesized *in vitro* with *E. coli* RNA polymerase (Boehringer-Mannheim) from 8000 NT heavy (*h*)-strand RSF2124 DNA template (2, 10). Enzymatic [^{3}H]RNA·DNA hybrids of sufficient length were selected by gel filtration on agarose A-15m (Bio-Rad) columns and by isopycnic centrifugation in CsCl/guanidinium chloride buoyant density gradients (11, 12). After denaturation, DNase I treatment, and deproteinization, light (*l*)-[^{3}H]RNA was recovered by Sephadex G-200 chromatography. The [^{3}H]RNA preparation used for this work had an average length of 850 NT, as determined by velocity sedimentation in denaturing gradients (4).

Hybridization Conditions and Data Reduction. Nucleic acids were denatured at 99° and incubated in 0.12 M phosphate buffer (PB)/0.2% sodium dodecyl sulfate at 60° or an equivalent criterion (6). As judged by sedimentation in denaturing gradients (4), degradation of RNAs did not occur during hybridization reactions. Reactions were assayed by HAP at 60° in 0.12 M PB/0.2% sodium dodecyl sulfate or at 40° in urea/phosphate (UP), by RNase treatment followed by Sephadex G-200 chromatography (13), or in CsCl/guanidinium chloride buoyant density gradients (11, 12). Various kinetic functions were required to fit the data as indicated in the *text*. Least squares solutions to the experimental parameters of these functions were obtained with the aid of a computer.

Empirical Expressions for DNA Excess Hybridization Kinetics. When excess double-stranded DNA is reacted with a single-stranded tracer, the rate of the tracer–driver hybridization reaction depends on the rate of disappearance of the single-stranded driver DNA. An empirical expression accurately describing the disappearance of single-stranded regions during the renaturation of randomly sheared DNA is

$$\frac{S}{C_0} = (1 + k_D C_0 t)^{-0.45} \qquad [1]$$

in which S is the concentration of NT in regions of single-stranded DNA at any given time t; C_0 is the total starting DNA concentration; and k_D is the second-order rate constant for the disappearance of totally single-stranded DNA molecules, as measured, e.g., by HAP assay. Eq. 1 was empirically obtained by Morrow (14) and ourselves (5) by measuring the appearance

Abbreviations: PB, phosphate buffer; HAP, hydroxyapatite; NT, nucleotides; C_0t, concentration of DNA (moles of NT per liter) × sec; UP, urea/phosphate buffer; RF, replicative form (double-stranded) of bacteriophage φX174 DNA; *h* and *l*, heavy and light DNA strand, respectively, of plasmid RSF2124 DNA.

* This is paper IV in a series. Paper III is ref. 1.

‡ Present address: Department of Biological Sciences, Simon Fraser University, Burnaby, British Columbia, Canada.

¶ Also Staff Member, Carnegie Institution of Washington.

of S1 nuclease-resistant DNA during renaturation. Some of the physical parameters that probably underlie the form described by Eq. 1 were analyzed in earlier papers of this series (5, 15). We showed there that the reactivity of single-stranded regions of DNA on molecules that also contain duplex structures is significantly less than that of free DNA strands of equivalent length. This inhibition can be expressed as a factor (E) modifying the nucleation rate constant and for the fragment lengths used here its value is about 0.5 (15). The inhibition factor is called E in the following derivation. We now consider the reaction of a tracer with excess DNA, in which the rate constant for the reaction of the tracer with the driver, k_R, is different from the rate constant for the driver DNA renaturation. The tracer, for example a labeled RNA, is present initially as single-stranded molecules whose concentration we shall term R_0, while R is the concentration of single-stranded tracer molecules remaining at any time in the reaction. The tracer may react with totally single-stranded driver molecules whose concentration is C, and also with single-stranded regions associated covalently with duplex regions. The concentration of these single-stranded sequences is $(S - C)$, where S and C have the same meaning as in Eq. 1. Thus, for the rate of tracer reaction,

$$\frac{dR}{dt} = -k_R CR - k_R ER(S - C) \qquad [2]$$

This expression is directly analogous to Eq. 3 of ref. 15. It is convenient to define a new variable, $V = 1 + k_D C_0 t$; thus $C = C_0 V^{-1}$. From Eq. 1, $S = C_0 V^{-n}$, where n equals 0.45. Differentiating V with respect to time and substituting in Eq. 2, we obtain

$$\frac{1}{R}\frac{dR}{dV} = -\frac{k_R}{k_D}V^{-1} - \frac{k_R}{k_D}E(V^{-n} - V^{-1}) \qquad [3]$$

A convenient form of solution to Eq. 3 is

$$\frac{R}{R_0} = (1 + k_D C_0 t)^{-k_R/k_D(1-E)} \times \exp\frac{k_R E[1 - (1 + k_D C_0 t)^{1-n}]}{k_D (1 - n)} \qquad [4]$$

Eq. 4 is used to fit the DNA excess hybridizations presented in Fig. 1 *A–E* of this paper. The values of k_R shown in Table 1 were extracted from the data by least squares methods, with n set at 0.45 (5) and E at 0.5 (15). The values of k_D listed in Table 1 and used for the determination of k_R were determined independently by least squares methods by application of a second-order function (Eq. 1 in ref. 5) to the driver renaturation data.

RESULTS AND DISCUSSION

Relative retardation of DNA-excess RNA hybridization

Fig. 1*A* displays the kinetics of hybridization reactions between excess double-stranded RSF2124 DNA and an asymmetric RNA tracer of similar length. Two independent methods of assaying the formation of RNA·DNA hybrids were used. The open circles show the binding to HAP of [^{3}H]RNA·DNA duplex. These reactions were analyzed in UP, which prevents binding of unhybridized RNA to the column while not affecting the binding of DNA·DNA duplexes (13). Table 1 shows that k_R for this reaction is 4.8-fold lower than k_D for the renaturation of the RSF2124 DNA. To determine whether this effect could be an artifact of UP/HAP assay procedure, a similar set of reactions was analyzed for hybrid formation by isopycnic sedimentation in CsCl/guanidinium chloride gradients (11, 12). In these gradients RNA, DNA, and RNA·DNA hybrids can all be resolved; in practice it was simplest to measure the decrease in the fraction of [^{3}H]RNA banding at the position of free RNA. As can be seen in Table 1, k_R for this reaction is indistinguishable from that obtained by the UP/HAP assay.

Over 90% of the [^{3}H]RNA was recovered in the RNA·DNA hybrid fraction in this experiment. We have observed this high level of termination only with the RSF2124 nucleic acids (Fig. 1 *A* and *F*), and in several experiments not included here in which the mean RNA fragment length exceeded 1500 NT. The RSF2124 RNA was about 850 NT long. We believe the ≤75% terminations often observed in DNA excess RNA hybridization (3–5) result from shorter RNA fragment lengths, as in the experiments shown in Fig. 1 *C–E*.

Fig. 1*B* shows that the retardation of RNA hybridization relative to DNA renaturation is also observed with *E. coli* nucleic acids. A third method of measuring hybrid formation is also shown in Fig. 1*B*. The reaction mixtures were treated with ribonuclease under conditions in which unhybridized RNA is destroyed but hybrids are spared. The hybridized [^{3}H]RNA was then detected by exclusion from Sephadex G-200 (13). While the kinetics of a reaction monitored in this manner are not strictly comparable to those monitored by isopycnic sedimentation or binding to HAP, it is obvious that the hybridization reaction is retarded relative to the driver DNA self-reaction. The complexity of the *E. coli* genome is about 400 times greater than that of the RSF2124 genome. It is striking that the ratio $k_D/k_R = 4.2$ for the *E. coli* reactions is nonetheless about the same as for the RSF2124 reactions. Note that the driver DNA concentration in Fig. 1*B* is up to 40 times higher than in Fig. 1*A*.

A third example is shown in Fig. 1*C*. This figure illustrates the kinetics of hybridization of a (+)strand RNA tracer with excess ϕX174 RF DNA as measured by the UP/HAP method. Table 1 shows that the rate constant for the DNA renaturation is in this case 3.0 times greater than that for the RNA·DNA hybridization. The *inset* shows an RNA excess hybridization reaction between exactly the same nucleic acids, reproduced from an earlier study (1). We showed there that the pseudo-first-order rate constant for the reaction of the RF [^{3}H]DNA tracer with excess (+)strand DNA is not distinguishable from the rate constant for the reaction of the same RF [^{3}H]DNA tracer with excess (+)strand [^{32}P]RNA driver.

Fig. 1 *A–C* demonstrates that regardless of the method of hybrid assay the hybridization reactions appear slower than the renaturation reactions even at the start. They do not begin at the DNA renaturation rate and then become progressively retarded. This argues against the possibility that the retardation is due to the buildup of DNA hyperpolymers formed as the driver renaturation reaction proceeds (15).

Effect of nonhomologous nucleic acids

In Fig. 1*D* an experiment similar to that illustrated in Fig. 1*C* is shown, except that a large excess of sea urchin rRNA was added to the reaction mixtures. Both the RF DNA renaturation rate and the hybridization rate were measured, as before. The purpose of this experiment was to determine whether the kinetics would shift to the nonretarded RNA excess rate when the total RNA concentration was brought to 40 times the DNA concentration. However, as Table 1 shows, neither k_D nor k_R was affected by the addition of rRNA. The *inset* in Fig. 1*D* describes a reciprocal experiment in which excess ϕX174 [^{32}P]RNA is reacted with RF [^{3}H]DNA tracer, but in the presence of a 200-fold excess of sea urchin DNA (with respect to the RNA). Again the nonhomologous nucleic acid fails to affect the

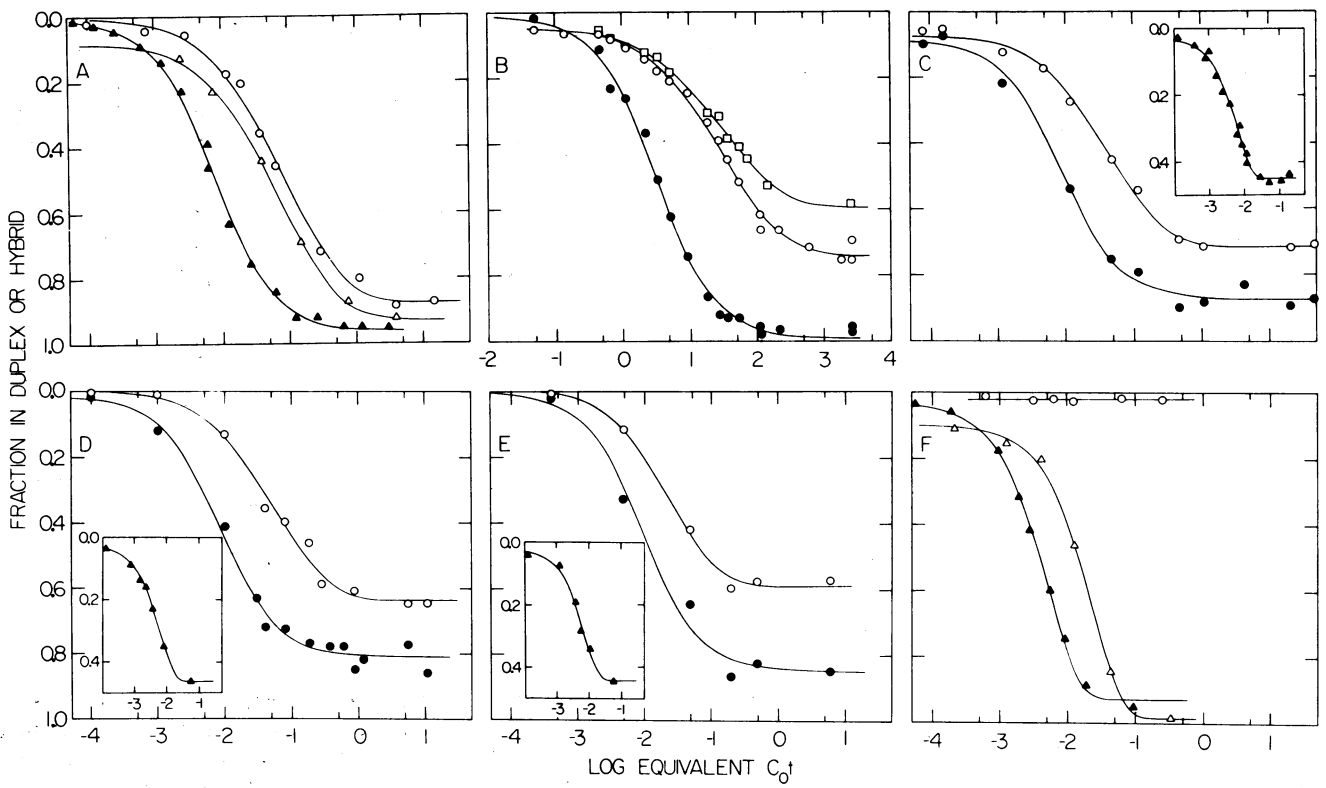

FIG. 1. Hybridization of various RNAs with excess DNA of similar fragment size. Except where noted the reactions were at 60°, 0.12 M PB criterion. DNA in duplex was assayed by HAP at 60° in 0.12 M PB (▲), or at 40° in UP (●). The renaturation of the DNA drivers (except in *F*) is fit with a second-order kinetic function (see ref. 5 for discussion). DNA hybridization with excess RNA shown in *insets* is fit with a pseudo-first-order kinetic function (Eq. 2 in ref. 1). RNA in hybrid was assayed by binding to HAP at 40° in UP (○) or by Sephadex G-200 chromatography following treatment with RNase (□), or by buoyant density centrifugation in CsCl/guanidinium chloride (△). The hybridization data (except in *F*) are fit by Eq. 4 in *text*. DNA concentrations and half-times for the hybridization reactions and rate constants derived from the least squares solutions are listed in Table 1. (*A*) Hybridization of 850 NT RSF2124 *l*-strand [^{3}H]RNA with a 3500-fold excess of unlabeled 1200 NT double-stranded DNA. The renaturation of 850 NT RSF2124 *l*-strand [^{3}H]DNA with a 20-fold excess of the same driver was followed in separate reaction mixtures. (*B*) Hybridization of 400 NT *E. coli* [^{3}H]RNA with a 150-fold excess of unlabeled 650 NT *E. coli* DNA driver. DNA renaturation was monitored by measurement of A_{260} in the HAP eluates. (*C*) Hybridization of 360 NT ϕX174 (+)strand [^{32}P]RNA with a 200-fold excess of unlabeled 300 NT ϕX174 replicative form (RF) DNA driver. Driver DNA renaturation was followed with 300 NT ϕX174 RF [^{3}H]DNA present in the same reaction at one-tenth the concentration of the [^{32}P]RNA. The *inset*, reproduced from the preceding paper of this series (1), shows the hybridization of 300 NT ϕX174 RF [^{3}H]DNA with a 40-fold excess of the same 360 NT ϕX174 (+)strand [^{32}P]RNA. The pseudo-first-order rate constant k_{pfo} for this hybridization reaction is 169 M^{-1} sec^{-1} (1). (*D*) Same as *C*, but with the addition of a 40-fold excess, with respect to the driver DNA, of 360 NT sea urchin rRNA. *Inset* as in *C* except addition of a 200-fold excess of 450 NT sea urchin DNA with respect to the RNA driver; for this reaction k_{pfo} = 160 M^{-1} sec^{-1}. (*E*) Same as *C* but incubated at 70°, 0.12 M PB criterion. The *inset* is the same as in *C* but the reaction mixture was incubated at 70° in 0.12 M PB; for the latter reaction k_{pfo} = 147 M^{-1} sec^{-1}. (*F*) Hybridization of 850 NT RSF2124 *l*-strand [^{3}H]RNA with a 400-fold excess of unlabeled 1200 NT RSF2124 *h*-strand DNA driver. The reassociation of 850 NT *l*-strand [^{3}H]DNA with a 20-fold excess of the same driver was monitored in separate reactions. Both sets of data are analyzed as pseudo-first-order functions, i.e., by use of Eq. 2 in ref. 1.

rate of the reaction. We conclude that the difference in the rates of DNA excess and RNA excess hybridization depends on the relative concentrations of complementary nucleic acids rather than on the total nucleic acid concentrations.

The experiments reviewed so far demonstrate that the relative retardation of the hybridization reaction is quantitatively similar over a range of two orders of magnitude in driver nucleic acid concentration and complexity (Table 1). It thus appears that this retardation stems directly from a decrease in the rate of nucleations through which complementary hybrid strand pairs are formed. This is also the implication of the observation noted above that the *initial rates* of the hybridization reactions are low relative to the driver DNA renaturation rate.

Temperature effect

In Fig. 1*E* is shown the kinetics of a DNA excess hybridization reaction employing the same ϕX174 nucleic acids as were used in Fig. 1 *C* and *D*. This reaction, however, was carried out at 70° rather than 60°, in the same ionic strength buffer. As expected, the rate constant for DNA renaturation is not changed by this temperature shift (Table 1). The terminal amount of hybridization is about 60% rather than the 65–70% observed in the other ϕX174 reactions. However, the rate constant for DNA-RNA hybridization at 70° is increased by a significant factor. Thus for the reaction of Fig. 1*E*, k_D/k_R is only 1.9. A similar result was reported earlier (16, 17). This result cannot be explained simply as an effect on the RNA, e.g., the unwinding of inhibitory secondary structure. The *inset* shows an RNA excess reaction also carried out at 70°. If anything, the rate of this reaction is slightly lower than for the 60° RNA excess reaction discussed above.

Pseudo-first-order DNA excess hybridization

A conceivable explanation for the retardation of hybridization with double-stranded DNA drivers is that some form of competition exists between the RNA and the other DNA strand for the transcribed strand. Such competition might have the net

Table 1. Approximate rates of hybridization of RNA with excess DNA of similar fragment length*

Nucleic acid (Fig.)	Length (NT) and strand			Hybridization			$C_0t_{1/2}$ RNA / $C_0t_{1/2}$ DNA	k_D†, M^{-1} sec^{-1}	k_R‡, M^{-1} sec^{-1}	k_D/k_R
	DNA driver	DNA tracer	RNA tracer	Driver DNA, $\mu g\ ml^{-1}$	Half-time, min	Assay				
ϕX174										
(1*C*)	300 RF	300 RF	360 (+)	40	4	UP	3.9	118	39	3.0
(1*D*, + rRNA)	300 RF	300 RF	360 (+)	40	5	UP	4.5	108	33	3.2
(1*E*, at 70°)	300 RF	300 RF	360 (+)	40	2.5	UP	2.1	109	58	1.9
RSF2124										
(1*A*)	1200 ds§	850 *l*	850 *l*	50	6.5	UP	8.6	133	28	4.8
					5.5	CsCl	7.3	133	30	4.4
(1*F*)	1200 *h*	850 *l*	850 *l*	9	9	CsCl	4.3	182¶	42¶	4.3
E. coli										
(1*B*)	650 ds§		400	150–1500	1.5–15	UP	8.0	0.32	0.071	4.2

* Reaction conditions are detailed in the legend to Fig. 1.
† Second-order rate constant for DNA driver renaturation derived by a least squares calculation using Eq. 1 in ref. 5.
‡ Least squares solution from Eq. 4 using listed k_D value.
§ Double-stranded (not strand-separated).
¶ Pseudo-first-order rate constant derived by a least squares calculation using Eq. 2 in ref. 1.

effect of reducing the frequency of successful DNA·RNA nucleations, and would of course not occur if the DNA were present only at tracer levels as in RNA excess reactions. The experiment shown in Fig. 1*F* was designed to test this hypothesis. RSF2124 DNA was strand-separated and sheared. Excess *h*-strand driver DNA was then reacted with the same *l*-strand tracer RNA as was used for the experiment shown in Fig. 1*A*, or with *l*-strand DNA tracer. The *h*-strand DNA cannot renature with itself and these reactions follow pseudo-first-order kinetics, as shown (1). Thus the values of k_R shown in Table 1 were obtained by the use of Eq. 2 in ref. 1. The DNA·RNA reactions could not be assayed by the UP/HAP method as shown in Fig. 1*F*. This is because DNA·RNA hybrids bind to HAP in UP only by virtue of covalently linked DNA·DNA duplex regions.‖ Instead isopycnic sedimentation was used to monitor hybrid formation. This experiment shows clearly that the hybridization reaction is retarded even with strand-separated DNA. Table 1 shows that the amount of retardation is just the same in this case as when double-stranded RSF2124 DNA driver is used. This result appears to rule out conclusively homologous DNA strand competition as an explanation for the relative retardation of the DNA excess hybridization reaction.

CONCLUSIONS

These studies show that the nucleation rate for RNA·DNA hybrid formation depends on which of the complementary nucleic acids is in excess. Our experiments were carried out with concentration ratios of DNA to RNA > 150, and we find that in standard conditions the rate constant for RNA·DNA hybrid formation is always 3- to 4.5-fold lower than the rate constant for DNA renaturation, or for RNA excess RNA·DNA hybrid formation.

This behavior represents a striking departure from the elementary expectation for ideal collision-dependent kinetics. At the concentrations employed there must be many collisions between complementary nucleic acids other than those resulting ultimately in formation of stable strand-pairs. A possible implication of the present results is that the occurrence of nonzippering associations between complementary strand pairs affects the rate of successful nucleations. This phenomenon may be effected by a rise in the temperature, and would depend on the ratio of RNA to DNA molecules in the reaction mixture. For example, the DNA molecules that ultimately form hybrids could participate in frequent interactions with complementary RNA fragments in RNA excess conditions, but not in DNA excess. Our main purpose here is to demonstrate as clearly as possible the empirical fact that the hybridization rate constant is reduced by a factor of 3 to 4.5 in DNA excess compared with RNA excess. Further investigation of this difference seems certain to yield new insights into the process of nucleic acid renaturation.

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‖ Because the DNA excess hybridization reactions in Fig. 1 *A–E* are all retarded to some extent with reference to DNA·DNA renaturation, most hybrid formation occurs anyway on particles containing already formed DNA·DNA duplex.

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