
Physical parameters affecting the rate and completion of RNA driven hybridization of DNA: new measurements relevant to quantitation based on kinetics

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ABSTRACT

Differences in the RNA-driven hybridization kinetics of genomic DNA and cDNA probes led us to examine physical parameters affecting these reactions. Cloned cDNA complementary to serum albumin (SA) mRNA hybridized in accordance with single component kinetics, whereas cloned SA genomic DNA hybridized more slowly and with multiple component kinetics. This difference is largely attributable to the relatively short and variable lengths of the mRNA complementary regions in the cloned genomic DNA.

The rate of mRNA driven hybridization is affected to about half the extent observed for DNA renaturation as Na^+ is increased or decreased from 0.18M.

In the annealing of nucleic acids of high sequence complexity, after ~70% of reaction has been reached, the rate of the reaction is slowed and completion is not reached under "static" conditions. In practical terms, this is not the case for systems of low sequence complexity. This problem can be largely overcome by continuous or frequent mixing of the reactants, so that complex cDNA probes are hybridized essentially to completion, and kinetics can therefore be more readily compared to simple complexity standards.

INTRODUCTION

RNA driven hybridization of single copy genomic DNA fragments of cDNA has been useful in estimating the sequence complexity of RNA in eukaryotic cells and tissues, and in measuring relative and absolute concentration of specific mRNA species. Much of what we know concerning the molecular genetics of cellular differentiation and function has been obtained from experiments involving nucleic acid hybridization. The application of nucleic acid hybridization to purification schemes, and to a wide variety of procedures and assays in recombinant DNA technology, is amply documented.

RNA driven hybridization of cDNA or single copy genomic DNA is a pseudo-first order reaction, the complexity of which is only partially understood. Because we have encountered several discrepancies in the kinetics of RNA driven hybridization in many of our experiments and those reported by others, we have investigated several physical parameters in an effort to better define features and conditions relevant to kinetics.

Here we present and evaluate data pertaining to the effect of 1) partial sequence discontinuity between genomic DNA fragments and mRNA, 2) Na⁺ (cation) concentration, 3) length of DNA tracers, and 4) continuous mixing, on the kinetics and degree of completion of mRNA driven hybridization of DNA. We show why anomalous kinetics have been observed in many instances, and describe conditions under which cDNA complementary to eukaryotic mRNA populations of high sequence complexity is hybridized essentially to completion.

METHODS AND MATERIALS

Preparation, and Sizing of RNA

Preparation of mouse brain or liver polysomes was as previously described (1,2), and poly(A)⁺mRNA was isolated by oligo(dT) cellulose chromatography (3). The size of various RNA preparations was determined by electrophoresis in denaturing agarose gels as described by Bailey and Davidson (4) as modified by Maxwell (5).

Isolation of Rat Serum Albumin Cloned Genomic and cDNA Sequences

Thirty micrograms of the SA genomic clone RSA 40 (representing all but the most 3' coding exon of the serum albumin gene, Sargent *et al.* (6), were digested with EcoRI yielding 7 fragments which were separated from the plasmid DNA by electrophoresis in a 1% agarose gels. Bands corresponding to 4.1, 3.1, 3.0, 1.8 and 1.3 kb were cut from the gel and the DNA was electrophoresed onto Whatman DE81 paper. The DNA was eluted from the DE81 discs with 2 M NaSCN, 0.05 M EDTA or 0.1 N NaOH and 0.05 M EDTA. DNA was precipitated with 2 volumes ethanol after addition of 10 µg yeast tRNA (directly from NaSCN or after neutralization of the NaOH elution mixture). The recovery of DNA by this procedure from the digestion reaction mixture was ~50% with NaSCN elution and 80% with NaOH elution.

To isolate cloned SA cDNA sequences, 10 micrograms of pRSA 12, a SA clone containing the 5' half of the coding sequence (~1100 nt) was digested with Pst I yielding one discrete band that migrated slightly faster than a 1221 nt marker in 1% agarose gels. The DNA was recovered as described above.

To label the isolated SA genomic or cDNA fragments, the DNA (0.5 to 2 µg) was denatured with NaOH, neutralized with HCl and Tris-HCl to pH 8.2 and added to a solution containing 50 µM ³H dCTP (23 Ci/mmol), 0.5 mM dCTP, 1.0 mM dATP, dGTP and TTP, 50 mM Tris-HCl (pH 8.2), 8 mM MgCl₂, 50 mM KCl, 0.12 A₂₆₀ units of random oligonucleotides, 8 units reverse transcriptase (J. Beard, Life Sciences, St. Petersburg, FL) in a total volume of 0.2 ml. The mixture was incubated at 37°C for 3 hours to yield DNA of an estimated specific activity of 2.5 to 3x10⁶ cpm per microgram.

DNA preparations were treated with 100 µg per ml proteinase K, extracted

with phenol and size fractionated on sepharose Cl-4B under alkaline conditions. Fractions containing DNA greater than 200 nt in length were neutralized and precipitated with ethanol after addition of yeast tRNA.

Synthesis of cDNA

Labeled cDNA complementary to mouse liver, brain poly(A)⁺mRNA, or encephalomyocarditis (EMC) viral RNA was synthesized using random oligo-deoxynucleotides or oligo-p(dT) as primers. Reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 8 mM MgCl₂, 4 mM Na pyrophosphate, 0.5 mM dithiothreitol, 0.4 mM each of dATP, dGTP, TTP, 10 μg actinomycin-D, 0.6 A₂₆₀ units of oligo-p(dT)₁₂₋₁₈ or 3.5 A₂₆₀ units of random oligodeoxynucleotides (7), 1 - 1.5 μg RNA, 15 units AMV reverse transcriptase and 2 x 10⁻⁵ moles ³H dCTP (approximately 25 Ci/mmole). All RNAs were held at 60°C for 3 minutes prior to addition to the reaction mixture. After incubation for 75 minutes at 37°C, the reactions were stopped by the addition of Na₂EDTA and SDS. *E. coli* RNA and phage fd DNA were added as carrier and each mixture was then extracted with phenol/chloroform pH 7.2. The cDNA was excluded from Sephadex G-100 in 0.1 M NaCl, 10 mM Tris-HCl (pH 7.4) and 1 mM Na₂EDTA. The RNA was hydrolyzed by placing the cDNA-mRNA duplexes in 0.1 M NaOH for 24 hours at 25°C. This mixture was then neutralized with HCl and the cDNA was precipitated by addition of ethanol and yeast tRNA.

Estimated transcriptional efficiency ranged from 65% to 95%. We have previously demonstrated that the sequence complexity of template RNA is conserved in the respective cDNA under conditions described above (2).

Electrophoresis and Sizing of cDNAs

"Full" length cDNAs were obtained only by priming with oligo-p(dT)₁₂₋₁₈ (2). Partial length cDNAs were generated by using random oligodeoxynucleotides as primers (see reference 2, for discussion). cDNA was sheared by release from a pressure cell equipped with a ball valve to a mass average size of about 350 nt.

The size of the cDNAs was determined by electrophoresis in alkaline agarose gels, (8). Fragments of SV40 DNA generated by digestion with HaeIII served as size references.

Annealing of Nucleic Acids

Hybridization or renaturation reaction mixtures using scDNA or cDNA as tracer (at concentrations as specified in the text) contained phosphate buffer (pH 6.8, equal molar mixture of mono- and dibasic sodium phosphate), 0.5% SDS, 5 mM Na₂EDTA and specified amounts of driver RNA or DNA. RNA driven hybridization was conducted in the presence of 0.18 M Na⁺ unless stated otherwise. Aliquots (0.5 to 100 μl) of the reaction mixtures were incubated in sealed capillary tubes at 60 to 68°C. Reaction aliquots were removed at various values of C₀t (moles of nucleotides

x time in sec.), rapidly frozen and stored at -20°C until assayed.

For continuous-mixing, 3-15 glass beads (diameter 0.11 - 0.25 mm) were placed into 5 or 10 μl capillary tubes (Corning pyrex micro-sampling pipets) containing 3 to 5 μl of the nucleic acid hybridization mixture. The capillary tubes were attached to a platform that was tilted to the left or right 70° from horizontal on a 2-3 min cycle. The platform was driven by a pneumatic ram fed through an electronically regulated air valve. The platform was housed in an oven maintained at 64° or 68°C .

For renaturation of cDNA or genomic DNA, C_0t values were corrected relative to Na^+ concentration according to tables constructed by Britten (9,10).

Preparation of mDNA

Genomic scDNA was hybridized to saturation by poly(A)⁺mRNA and hybrids were isolated by hydroxyapatite chromatography. The isolated duplex molecules were divided into two fractions. One fraction was digested with RNAase in low salt to "release" the hybridized DNA (Galau *et al.* (11), as modified by Bantle and Hahn (12). The other fraction was digested first with S_1 nuclease and, after removal of S_1 nuclease, with RNAase. The preparation was again passed through HAP to remove the small amount of DNA-DNA duplexes which had formed by self renaturation of scDNA. These two preparations are referred to as HAP-mDNA or HAP- S_1 nuclease mDNA respectively. mDNA preparations were exhaustively deproteinized using proteinase K and phenol extractions.

Assay of RNA/DNA Hybrids or DNA/RNA Duplexes

Hybrids and duplexes were assayed by the S_1 nuclease/DEAE-81 filter method (13) as modified by Van Ness *et al.* (1). If the nucleic acid level was low in the assay reaction, as in the case of EMC cDNA hybridization, sheared *E. coli* DNA, or RNA was added (0.05 $\mu\text{g}/\mu\text{l}$) to reduce loss due to nonspecific binding to vessel walls.

When the hybrids or duplexes were assayed by hydroxylapatite chromatography the procedure used was as previously described (12).

RESULTS AND DISCUSSION

Effect of Sequence Discontinuity and Length of Complementary Sequence of the Tracer on the Rate of Hybridization

We have observed marked differences in the kinetics of mRNA driven hybridization of scDNA and cDNA (representing complex class mRNAs) tracers of similar length. In brief, we have found that fragments of scDNA hybridized much more slowly than expected (2). The difference between the hybridization kinetics of fragments of genomic DNA and cDNA might be due to the presence of intervening sequences and/or the relatively short runs of complementary sequences (exons) in the genomic DNA fragments. We have used cloned genomic DNA and cDNA complemen-

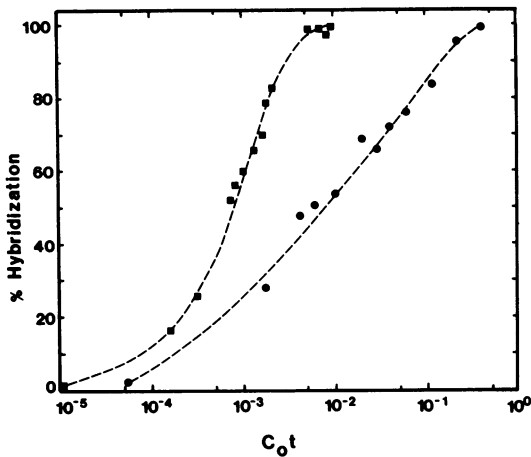


Fig. 1. Hybridization of cDNA (■) and genomic DNA (●) complementary to rat serum albumin mRNA. cDNA was from clone pRSA-13 and genomic DNA was from clone RSA-40 (Sargent *et al.*, 1979). After shearing, cDNA and fragments of genomic DNA were ~450 nt mass average size. Reactions are normalized to 100%. Actual % hybridization of the cDNA (1/2 of which is complementary to mRNA) was 48% and 7.8% for the genomic DNA. Based on mapping data of the cloned genomic albumin DNA, 7.0% of the total genomic DNA was expected to be complementary to SA mRNA. C_0t is calculated on the basis that 14% of the mass of mRNA was SA mRNA.

tary to serum albumin mRNA as a model system to test these possibilities.

Fig. 1 shows the hybridization of cloned cDNA and genomic DNA complementary to rat serum albumin (SA) mRNA. cDNA complementary to the 5' half of the SA mRNA (sheared to a mass average length of ~400 nt) hybridized in accordance with single component pseudo-first order kinetics. The rate of hybridization indicated that ~14% of the total poly(A)⁺mRNA is composed of SA mRNA, a value similar to that previously obtained by Tse *et al.*, (14). Hybridization of cloned genomic DNA (sheared to a mass average size of ~450 nt) was about 10 times slower to half reaction and extended over 3.5 C_0t decades. Completion of the reaction occurred at a value of C_0t 80 times greater relative to that of the cDNA tracer.

Since all other parameters of the reactions were identical, the difference in the rate of hybridization is attributable only to differences between the DNA tracers. The slower rate of hybridization of the cloned genomic DNA is apparently not due to some unknown factor which interferes with base pairing, since cloned cDNA and genomic DNA renatured in accordance with their respective complexities (not shown). cDNA molecules are fully complementary to SA mRNA, whereas most of the genomic DNA fragments which hybridize are a mix of complementary (code) and noncomplementary (intervening) sequences. Since the average length of the coding regions (exons) in the SA gene is ~120 bp (6) and the mass average size of the tracer molecules is 450 nt, most of the randomly sheared genomic DNA fragments containing all or part of a coding sequence also contain intervening sequence (IVS) DNA. Also about two-thirds of these molecules would be expected, statistically, to

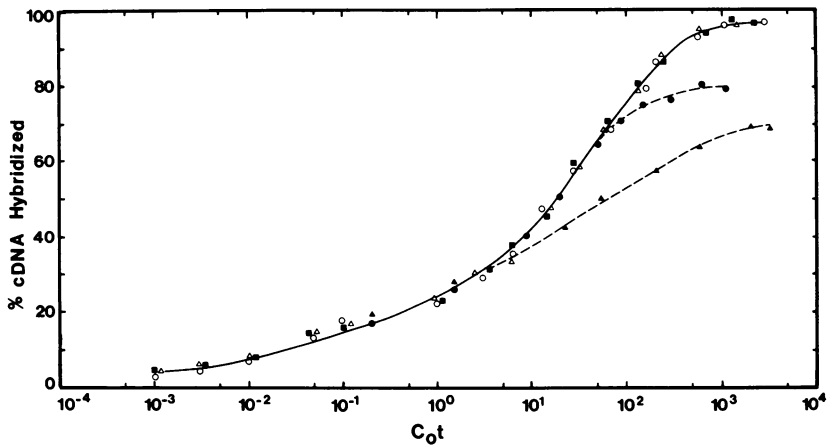


Fig. 2. Liver mRNA driven hybridization of respective cDNA of different mass average lengths. (\blacktriangle), ~120 nt; (\bullet), ~250 nt; (\circ), 400 nt; (\square), 800 nt; and (Δ), 1200 nt. Mass average size of mRNA at $t=0$ was ~1300 nt and ~700 nt after $C_0t 10^3$. Mass of mRNA to cDNA in the hybridization mixtures was 10,000:1.

contain segments of IVS at each end constituting, in sum, ~65% of the total length.

Considering these characteristics of the SA genomic DNA fragments, two separate or combined mechanisms may cause the rate retardation we observed. First, zippering leading to a stable hybrid following nucleation may be aborted with greater frequency due to the presence of noncomplementary sequences, and thus more nucleation events would be required. Second, the observed rate of reaction is known to be affected by the length of complementary sequence within the DNA fragment (15,16,17). As shown in Fig. 2, when the length of the complementary sequence is ~120 nt or less, the rate and completion of hybridization is significantly less than when the modal length is > 120 nt. Hence the cloned genomic DNA tracer would be expected to hybridize more slowly than the cDNA (Fig. 1), since most of the hybridizing fragments of genomic DNA contain a complementary sequence which is shorter than the length of the average exon, (i.e. < 120 nt).

The fact that the hybridization of the SA genomic DNA was not completed within the 1.5 - 2.0 C_0t decades expected for a single component pseudo first order system, but rather is spread out over more than 3 C_0t decades (Fig. 1), is probably a reflection of the variability of lengths of complementary sequences in the DNA fragments. The length of the exons in the cloned DNA is between about 60-250 nt sequences and random shearing adds to the variability of the length of these sequences. Hence hybridization is extended over several C_0t decades with the bulk of shorter sequences likely hybridizing more slowly than the longer sequences.

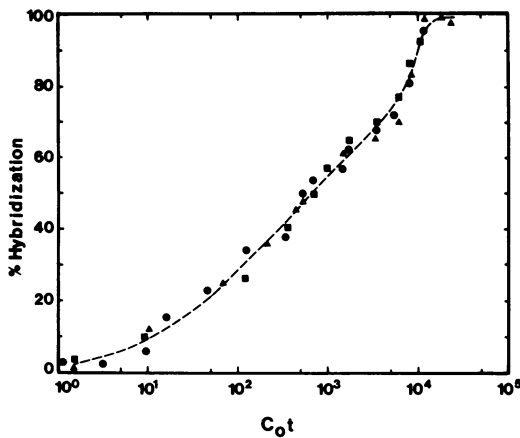


Fig. 3. Liver mRNA (same preparation as used for experiment in Fig. 2) driven hybridization of various single copy genomic DNA probes. (▲), total scDNA; (●), mDNA HAP; (■), S₁ nuclease-mDNA. For comparative purposes, reactions were normalized to 100%. Absolute hybridization of total scDNA was 2% (also see Ref. 2), 58% for mDNA-HAP, and 80% for mDNA prepared by the S₁ nuclease-HAP method (see Methods). Mass average size of total scDNA, mDNA-HAP and S₁-nuclease-HAP mDNAs, was 400, 320, and 190 nt respectively.

Of these differences between cDNA and genomic DNA tracers (continuity and length of complementary sequences) the length of the complementary sequence apparently has the far greater effect on the rate of hybridization. We tested this in the experiment shown in Fig. 3, where three types of scDNA tracers are compared. One tracer is single copy mouse genomic DNA recovered from hybrids isolated by HAP chromatography after saturation hybridization with liver mRNA, and the other tracer is a portion of the hybridized scDNA which was digested with S₁ nuclease to remove unhybridized sequences (intervening or flanking sequences) to yield "mDNA" or DNA fully complementary to mRNA (see Methods). These tracers, which are expected to have continuous complementary sequences of the same length, hybridized with liver mRNA at a similar rate (Fig. 3). Hence, the presence of noncomplementary sequences (intervening or flanking) in scDNA tracers of mass average size of 320 nt does not grossly affect the rate of hybridization. Therefore we conclude that the slower rate of hybridization of the cloned genomic DNA relative to cDNA (Fig. 1) is largely attributable to the fact that the mRNA complementary sequences in the genomic DNA fragments are much shorter than the cDNA.

It has been previously demonstrated that the rate of a second-order hybridization or renaturation reaction is proportional to the square root of the molecular weight of the RNA or DNA reactant species (18,19,20). The length of the short strand was found to determine the rate of hybridization between long RNA as driver and short DNA as tracer (21). Also, the length dependence for driver-tracer reassociation rates is greater when reacting strands are of the same average length (22).

As a matter of practical importance we examined the rate of hybridization of cDNA of various lengths. As shown in Fig. 2, cDNAs of mass average length of 400-

1200 nt hybridize with similar kinetics. Also when cDNA of mass average size of 3200, 1550 and 800 nt complementary to EMC virus RNA was used (not shown) no apparent difference in the kinetics of hybridization of these probes was observed. However, cDNAs of 120 and 250 nt hybridize more slowly (bear in mind that many of the cDNA molecules are much smaller than the average size.) In this regard the "plateauing" observed for the 120 and 250 nt tracers, which occurs before completion of the reaction, might be due, in part, to the lower thermal stability of hybrids involving cDNA fragments which are considerably smaller than the mass average size. During the course of hybridization cDNA tracers were not appreciably reduced in size. The mass average size of the liver mRNA was ~1300 nt at the start of the hybridization, but owing to modest cleavage during incubation mass average size was reduced to ~700 nt after the longest incubation period which was used. The important point of these experiments is that the hybridization of cDNA in the size range of 120 - 250 nt differs considerably from that of cDNA in the 400 - 1200 nt range when driven by the same mRNA. These results show that it is desirable to use cDNA tracers which are ~400 nt in length, if kinetics and completion are important, as in sequence complexity measurements.

Several factors probably influence the degree to which a length effect is observed such as the method of duplex assay, the species (DNA or RNA) in excess, the starting size of the reacting molecules and the disparity of the lengths of the RNA and DNA fragments in question. Our purpose here has not been to quantitate this phenomenon in detail, but to point out practical constraints which should be placed on cDNA tracer size to decrease variability in kinetics. In brief, we conclude that the length effects which apply to DNA reassociation are not directly applicable to RNA driven hybridization as assayed by resistance to S_1 nuclease, and that there is little difference in kinetics of hybridization of cDNA ranging in size from 400 to 3000 nt (mass average size).

We conclude that when the length of complementary sequences in the hybridizing genomic DNA fragment is short, the rate and extent of hybridization is decreased. Detailed structural analysis of several mammalian genes shows a range of exons between 30 and 1200 nt (6,23,24). Since individual exons are postulated to correspond to structural or functional domains of proteins (25,26), it may well be that the average exon length in the mammalian genome is 200 nt or less. Therefore the kinetics of mRNA driven hybridization of scDNA might vary in relation to the sequence organization of the genome in question.

Monovalent Cation Concentration and Rate of RNA Driven Hybridization

Previously, values for the effect of monovalent cation concentration on rate of DNA renaturation, (9,10), have been applied to RNA driven hybridization of labeled

TABLE 1.

Nucleic Acid	[Na ⁺]	K L/Mol ⁺ /sec	Expected relative rate (a)	Observed relative rate (b)	Expected/ observed
EMC RNA/cDNA	0.09	58	0.18	0.50	0.36
	0.18	115	1.00	---	---
	0.60	230	4.90	2.0	2.4
E. coli RNA/DNA	0.09	0.077	0.18	0.36	0.5
	0.18	0.215	1.00	---	---
	0.60	0.575	4.90	2.66	1.8
Ascites poly(A) ⁺ mRNA/cDNA	0.18	0.135	1.00	---	---
	0.60	0.328	4.90	2.42	2.0
Brain poly(A) ⁺ mRNA/cDNA	0.18	0.054	1.00	---	---
	0.60	0.132	4.90	2.40	2.0
E. coli DNA (renaturation)	0.09	0.007	0.18	0.22	0.8
	0.18	0.031	1.00	---	---
	0.60	0.142	4.90	4.60	1.0
Mouse single copy DNA(renaturation)	0.18	0.00015	1.00	---	---
	0.60	0.00076	4.90	5.06	1.0

DNA tracers used for the studies were 300-400 nt in length and mass average size of the RNA was ~1000 nt or greater. Rate constants for *E. coli* RNA driven hybridization pertain to the infrequent class mRNA species complementary to ~60% of the sense strand equivalent of the genome (see ref 29). Rate constants for mouse ascites and brain poly(A)⁺mRNAs are based on the overall hybridization reaction since effect of [Na⁺] is the same for all copy frequency components. Hybridization or renaturation was measured by the S₁ nuclease-DEAE filter method (13).

- (a) Expected relative rate is from tables constructed by Britten for DNA renaturation (9,10).
 (b) Rate observed is relative to that in 0.18 M Na⁺ which is taken as 1. Values given are the average of 3 or more determinations for each system.

DNA fragments, although it had not been shown that these values are applicable in this case (9). We observed that the relative increase in rate of mRNA driven hybridization due to increasing Na⁺ concentration above 0.18M is approximately one-half of that observed for DNA reassociation.

We used viral, bacterial and mammalian nucleic acids to compare the effects of [Na⁺] on RNA driven hybridization of DNA and DNA reassociation. Rate constants for each of six systems we used are given in Table 1. Values for rate constants (K) were calculated from Cot1/2 values or obtained from plots prepared by the method of Jacquet *et al.*, (27). Our observed values in the case of DNA renaturation are in close accordance with those given in tables constructed by Britten (9,10).

As in the Britten tables we have used 0.18M Na⁺ as the standard (i.e., relative rate equals 1.0 at that Na⁺ concentration). Above 0.18M Na⁺ concentration, the relative rate of RNA driven hybridization of DNA is, in general, affected to about half the extent observed for the relative rate of DNA renaturation. Below 0.18 Na⁺, the relative rate of RNA driven hybridization is decreased less than that for DNA renaturation. Thus the relative rate of RNA driven hybridization, for mRNAs in general, is affected less by varying Na⁺ concentration than is DNA renaturation.

All RNAs used for these studies were passed through Sephadex (G-100 or G-50) and used directly in hybridization to insure accuracy in Na⁺ concentration (28). This was important because we have observed, using ²²Na as a monitor, that when RNA is precipitated by addition of ethanol, 2 x 10⁻⁸ to 4 x 10⁻⁸ mole of sodium is precipitated per μg of RNA (Van Ness and Hahn, unpublished observations). This co-precipitation of Na⁺ is independent of Na⁺ concentration in solutions of RNA over the range of 0.05 to 0.50 M NaCl and RNA concentrations between 10 μg/ml to 100 μg/ml. Thus, for example, if 100 μg of RNA were precipitated in the presence of Na⁺ by addition of ethanol, and then redissolved in hybridization buffer at 5 μg/μl (a concentration suitable for hybridization in the case of complex mRNA populations) the co-precipitated Na⁺ alone would yield a concentration of 0.1 -0.2M.

From empirical data over the range of Na⁺ we used, a table for estimating the relative rate of RNA driven hybridization relative to Na⁺ concentration was constructed (Table 2). Two separate power functions were applied to best fit, by nonlinear-regression, the data which we used to obtain the values given in Table 2. The function $n = K^{0.05K}$ best fits the relationship of sodium versus rate constant in the monovalent cation concentration range of 0.03 to 0.18 M, where n equals the relative change in reaction rate at the specified monovalent cation concentration (K) relative to that in 0.18 M Na⁺. The second equation $n = K(0.2/K)^{0.56}$ best fits our data for concentrations of 0.18 M Na⁺ and above. The relative rates in Table 2 are based on 0.18 Na⁺ as the standard value of 1.0, as per tables constructed by Britten pertaining to DNA renaturation.

The values are for conditions ~20°C below melting temperature at the specified Na⁺ concentration, but are probably valid to ± 10°C of the optimum temperature, since the temperature optimum range for RNA driven hybridization is wider than that observed for DNA renaturation. For example, the rate of RNA driven hybridization of T4 DNA is retarded by only 15% at ± 14°C of the optimum incubation temperature (15), whereas renaturation of DNA is reduced by 30% - 40% at ± 14°C of the optimum temperature (30). Additionally, we observed no apparent difference in the rate of RNA driven hybridization of *E. coli* DNA at 60° and 68°C in the presence of 0.18 M Na⁺.

TABLE 2.

Rate of mRNA Driven Hybridization Relative to $[Na^+]$					
Molarity Na^+	PB ^(a) Molarity	Relative Rate	Molarity Na^+	PB ^(a) Molarity	Relative Rate
0.10	0.067	0.52	0.28	0.186	1.74
0.11	0.073	0.60	0.30	0.200	1.83
0.12	0.080	0.66	0.32	0.218	1.91
0.13	0.087	0.74	0.34	0.227	2.00
0.14	0.093	0.79	0.36	0.240	2.06
0.15	0.100	0.85	0.38	0.253	2.14
0.16	0.107	0.92	0.40	0.267	2.20
0.17	0.113	0.97	0.42	0.280	2.26
0.18	0.120	1.00	0.44	0.293	2.31
0.19	0.127	1.05	0.46	0.306	2.37
0.20	0.133	1.08	0.48	0.320	2.40
0.22	0.146	1.31	0.50	0.333	2.46
0.24	0.160	1.49	0.55	0.366	2.54
0.26	0.173	1.60	0.60	0.400	2.60

(a) PB is phosphate buffer containing equimolar amounts of mono- and disodium phosphate.

The mechanism(s) whereby RNA driven hybridization of DNA and DNA renaturation are differentially affected by Na^+ concentration are unknown. It is likely that the difference in the hydration schemes of A-RNA and B-DNA, owing largely to the 2' hydroxyl group of ribose permitting 1/3 more Na^+ into the hydration shell, is an important physical consideration (31). The hydration shell for RNA is more stable than for single strand DNA as ionic conditions are varied. Therefore, the physical state of RNA in aqueous solution is probably less affected as ionic conditions are varied (within a limited range) than is single strand DNA. Whatever the mechanism(s), the difference in the effect of Na^+ concentration on RNA driven hybridization versus DNA renaturation is not due to differences in the stability of RNA/DNA versus DNA/DNA duplexes (32-36). Also the differences are probably not attributable to differences in nucleation (at least under standard conditions) between RNA driven hybridization and DNA renaturation, since the rates for these events are essentially the same for both reactions (15,37).

Our results pertain to reactions driven by mRNA, or probably, more generally, to RNAs with comparatively little secondary structure. Hybridization driven by tRNA and rRNA or other RNAs with extensive secondary structure may be affected less by ionic concentrations. For example, we have observed that rRNA driven hybridization of respective cDNA (cDNA synthesized by the random oligonucleotide method, (7) occurs at essentially the same rate in the presence of 0.18, 0.24 or 0.6M

Na⁺.

We do not know whether the table we have constructed also applies to DNA driven hybridization of RNA. However, the physical chemistry of RNA in aqueous solution applies whether RNA is the tracer or the driver, and therefore the table we have constructed probably applies in this case.

Effect of Continuous Mixing on the Rate and Extent of Completion of DNA Renaturation or Hybridization

We present empirical results which show that continuous mixing of reactants alters the kinetics and, in particular, the degree of completion of hybridization reactions in the case of nucleic acid systems of high sequence complexity. Without continuous mixing, complex cDNA probes fail to be completely hybridized within a practical time frame, which in theory should be achievable. In contrast, in simpler systems (viral or bacterial nucleic acids), the theoretical expectation of 100% hybridization is essentially achieved using the same procedure (i.e., reaction mixtures contained within sealed capillary tubes, duplexes assayed by resistance to S₁ nuclease).

We noticed the failure to achieve complete reaction with mammalian cDNA-mRNA systems was particularly apparent when the amount of cDNA (tracer) to mRNA (driver) was increased, as in the case of preparative hybridization used to modify the composition of complex cDNA probes, i.e. in the preparation of null probes. This is shown in Figure 4 in which different tracer/driver ratios have been used. In this case using a mouse liver poly(A)⁺mRNA-cDNA system, a plateau occurred after ~ 72% reaction when a 75:1 ratio of mRNA to cDNA was used, despite the fact that less than 2% of the mRNA would be base paired with cDNA upon complete hybridization of the cDNA. Even at the 7500:1 ratio, which we often use to obtain analytical hybridization curves, a plateau in the reaction occurred before the cDNA was completely hybridized (Fig. 5). In contrast, cDNA in the EMC viral RNA-cDNA system was hybridized essentially to completion in all instances in which the mass of RNA to cDNA was 10:1 or greater (Fig. 5).

When solutions were mixed continuously during the course of the reaction (see Methods), 98% of the cDNA was hybridized in mixtures where the ratio of mRNA/cDNA was 75:1, in comparison to 72% hybridization when mechanical mixing was omitted during the course of the reaction (Fig. 4). In the case of a high ratio of mRNA to cDNA (7000:1) continuous mixing also alters the last 1/3 of the reaction, and hybridization of cDNA complementary to rare mRNAs is observed to approach completion (>98%, Fig. 5). We emphasize that the extent to which reactions approach the expected level of completion (100%) is affected more than are the kinetics of that portion of the reaction which was observed to occur.

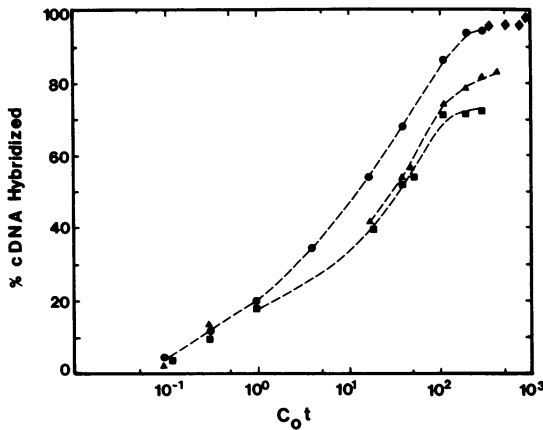


Fig. 4. Liver mRNA driven hybridization of total respective cDNA at various mass ratios of mRNA to cDNA under "static" conditions. \blacksquare , 75:1; \blacktriangle , 750:1; \bullet , 7500:1. Termination is reached at mRNA/cDNA = 75:1 under conditions of continuous mixing, \blacklozenge . Concentration of mRNA in all cases was $6 \mu\text{g}/\mu\text{l}$ for C_0t values above 10.

Notice in Fig. 5 that hybridization of cDNA complementary to highly abundant and intermediate copy frequency classes of liver mRNA is not altered by continuous mixing when mRNA is in vast excess (in this case mass ratio was $\sim 3500:1$). In this case there is much lower complexity and hence higher relative concentration of each

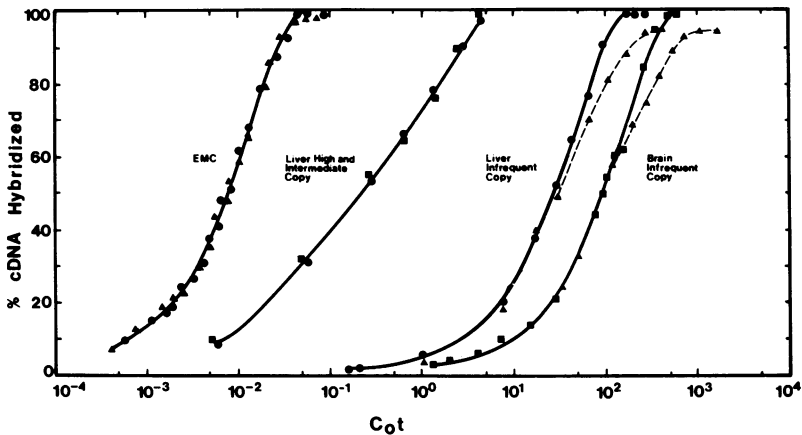


Fig. 5. RNA Driven Hybridization of cDNA. Effect of Continuous Mixing in Relationship to Complexity. The 4 curves at far right show the hybridization of cDNA complementary to infrequent copy liver or brain poly(A)⁺mRNA in the presence (solid lines) or absence (dashed lines) of continuous mixing. Curve in center shows hybridization of cDNA complementary to high and intermediate copy frequency liver poly(A)⁺mRNA in the presence (\blacksquare) or absence (\bullet) of continuous mixing. At far left, is hybridization of EMC virus cDNA with (\bullet) or without (\blacktriangle) continuous mixing. mRNA to cDNA ratios for mouse liver and brain systems was 7000:1 in the case of the infrequent probe and 3500:1 for the probe complementary to high and intermediate abundance class mRNA. For the EMC system RNA/cDNA was 100:1.

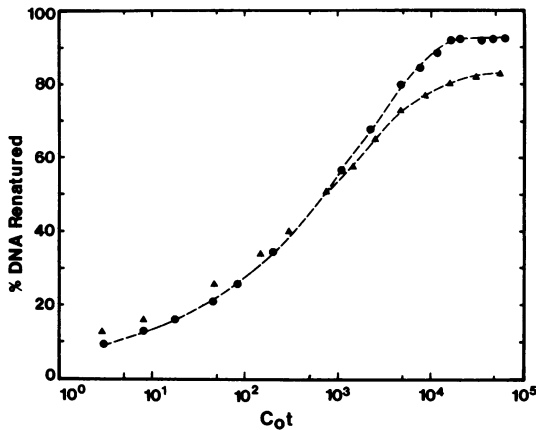


Fig. 6. Renaturation of fragments of mouse genomic DNA in the presence (●) or absence (▲) of continuous mixing. Mass average size of tracer and driver DNA fragments was ~400 nt. DNA is ~80-85% single copy DNA. Renaturation was measured by HAP chromatography. Binding efficiency of 400 nt duplexes to HAP was ~98%; values shown are not corrected for this slight chromatographic inefficiency. Concentration of DNA was 7 $\mu\text{g}/\mu\text{l}$. Renaturation was conducted at 68 $^{\circ}$ C in the presence of 0.6 M Na $^{+}$.

of the sequences (the combined complexity of abundant and intermediate class species is ~1200 kb). Likewise, the effect of continuous mixing on the EMC RNA-cDNA system (complexity ~9.7 kb) is not observed at the concentration of nucleic acids which are routinely used (Fig. 5).

It is clear that complexity estimates based on kinetics of cDNA hybridization, even at high ratios of mRNA to cDNA, in the absence of continuous mixing are difficult to directly determine via reference to a simple complexity standard, particularly if kinetics are analyzed by useful methods such as described by Jacquet *et al.* (27). However the C_0t 1/2 method of determining complexity relative to a simple standard appears to be fairly satisfactory if a high ratio of mRNA to cDNA (~7000:1) is used as shown in Fig. 5.

As expected, DNA renaturation, a second order reaction, is also affected by mixing. Fig. 6 shows renaturation of genomic DNA (mostly "single copy" DNA) in which continuous mixing alters the kinetics and extent of renaturation. Previously, the extent of renaturation of DNA has been used by several investigators to determine the "availability" of DNA tracer used in saturation hybridization experiments for measurement of sequence complexity of RNA populations (11,12,38,39). In the absence of continuous mixing, the extent of renaturation of a labeled DNA tracer, prepared from DNA of high sequence complexity, can lead to an underestimation of the potential reactivity (hybridizability) of the probe, although in many cases this is of minor consequence.

The physical chemistry of the annealing of nucleic acids in solution is not well understood, but it is clear that in complex systems millions of collisions between polynucleotides must occur on average (most of them between non-complementary molecules) before complementary sequences become base paired. Thus the diffusion

and the relative elasticity of the collisions between polynucleotides are parameters which bear upon the annealing of nucleic acids in solution. Schmitz and Schurr (40) have argued that renaturation of DNA is controlled by rotational diffusion in that the stringent orientation of molecules is required before nucleation can lead to annealing. They presented the view that diffusion is a far greater factor in control of rate than is excluded volume (steric hinderance) as proposed by Wetmur and Davidson (18). They concluded that the elementary rate constant is determined by rotational diffusion.

Several interpretations of our observations on the effect of continuous mixing are possible, but it is beyond the scope of this paper to present detailed considerations of relevant theoretical aspects. The fact that mixing does not alter, in any apparent manner, the kinetics or extent of completion of reactions in the case of simple systems, but does in complex systems, suggests that processes, other than those accounted for by the elementary rate constants, should be considered. One possibility is that mixing aids in the translation diffusion of molecules. In complex systems, owing to the much higher required concentration of nucleic acids and the greater number of collisions between non-complementary sequences occurring, on average, per collision between complementary molecules, the parameter of translation diffusion, viewed in terms of the statistics of random walk of macromolecules (41) is of greater magnitude than in simple systems. Another aspect is that movement of the beads in the capillary tubes might generate shearing forces which are sufficient to disrupt or speed up the disruption of aggregated molecules, thus freeing polynucleotides which might have been unavailable for hybridization. However, we cannot align this possibility with the results shown in Fig. 4. Regarding Fig. 4, the statistics of random walk serve to at least partially explain our result, in that the probability of a tracer DNA molecule encountering a complementary RNA molecule is proportional to the number of such RNA molecules which, statistically, have a good probability of colliding with the DNA at any given instant.

Whatever the possible interpretation, our results show that in complex systems, continuous mixing of reactants during the course of renaturation or hybridization, largely eliminates the "false" termination of reactions and has modest effect on kinetics. Regarding this latter point, Kohne *et al.* (42) have shown that the rate of annealing is greatly increased when the reaction mixture is maintained in a phenol-water emulsion by rapid and continuous mixing. Thus, when mixing is continuous or frequent, the rate of renaturation for the overall reaction of DNA of high sequence complexity, is inversely proportional to complexity, and the reaction rate is dependent upon the initial concentration of reactants, as described by Wetmur and Davidson (18) and Britten and Kohne (43). Therefore, we suggest that, when

necessary, reactions be performed under conditions where molecules are mixed continuously. This is useful in the preparation of "null" or "positive" probes since recycling of the probe through the purification scheme can be eliminated or minimized.

Mixing (Figures 4 and 5) is important when complexity estimates are obtained from the hybridization kinetics of cDNA in the case of complex systems, particularly when the ratio of driver RNA to cDNA is relatively low (Fig. 4). In the case of complexity estimates based on saturation hybridization of single copy genomic DNA, continuous mixing is likely of less consequence since these estimates do not rely upon kinetics, and by design a high driver to tracer ratio is generally used.

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