RNA-DNA Hybridization: Demonstration in a Mammalian System of Competition by Preincubation

Abstract. *Nonspecific intermolecular interactions obscure the interpretation of competition experiments involving hybridization of DNA and RNA in mammalian systems when the labeled RNA/DNA ratio is low. The effect of these interactions can be minimized by choosing a sufficiently high ratio of labeled RNA/DNA. No serious effect of nonspecific interactions is observed in bacteriophage systems, even at very low labeled RNA/DNA ratios.*

Competition among various RNA's for binding sites on DNA has been used widely to demonstrate the similarity or dissimilarity of different RNA preparations (*1*). Recently, Birnboim and co-workers (*2*) cast doubt on the interpretation of such experiments with mammalian nucleic acids. They found that unlabeled RNA was unable to reduce binding of labeled RNA to nitrocellulose discs loaded with DNA when the unlabeled RNA was incubated with the discs and washed off, and the discs were then exposed to labeled RNA. By using a much larger concentration of RNA (relative to DNA), we have been able to demonstrate competition in a mammalian system by the preincubation technique. When we used RNA concentrations comparable to those used by Brinboim and co-workers, we too failed to observe competition by the preincubation technique.

The preincubation technique was first used by Kasai and Bautz (*3*), who demonstrated that unlabeled bacteriophage RNA, when incubated with DNA-loaded nitrocellulose discs, would occupy specific sites on the DNA, and that these sites would not be available for binding labeled RNA incubated with the discs after they were washed. Subsequently, Oda and Joklik (*4*) successfully used preincubation with vaccinia virus nucleic acids. Birnboim *et al.* postulated that, in mammalian systems, the unlabeled RNA interferes with hybridization of labeled RNA by a mechanism which does not depend on the occupation of specific DNA sites by identical molecules.

Other than the sources of the nucleic acids, the most conspicuous difference between the mammalian experiments and the virus experiments is the ratio of the various nucleic acids used. Kasai and Bautz (*3*) used saturating or near-saturating amounts of RNA, as did Oda and Joklik (*4*); Birnboim *et al.* (*2*), on the other hand, used concentrations of labeled RNA far below the saturating levels. In order to determine whether the relative nucleic acid concentrations were critical for the success of the experiment, we carried out competition experiments (*5*) with mammalian nucleic acids at both high and low concentrations of labeled RNA. At low concentrations of RNA (Table 1, groups 1 and 2) we observed apparent competition only when labeled and unlabeled RNA were incubated with the DNA simultaneously. However, when we increased the amount of RNA 50-fold, to saturating levels, we observed competition both when the RNA's were added simultaneously and when the DNA-loaded discs were preincubated with unlabeled RNA.

In saturation experiments using increasing amounts of labeled RNA, we established that saturation occurs in this system, and under these conditions, at between 150 and 200 μg of RNA input. We have chosen to use 200 μg of RNA in our saturation experiments, although we would expect to obtain qualitatively similar results at near-saturation levels of 100 or 150 μg.

Table 1. Group 1: hybridization of 4 μg of ^{3}H-labeled L-cell RNA to 2 μg of mouse DNA in the presence of varying amounts of unlabeled L-cell DNA. Input label, 3×10^4 count/min. Group 2: same as group 1, except that unlabeled RNA was added first, washed off, and then labeled RNA was added. Group 3: hybridization of 200 μg of labeled L-cell RNA with 2 μg of mouse DNA in the presence of varying amounts of unlabeled L-cell RNA. Input label, 6.8×10^5 count/min. Group 4: same as group 3, except that discs were exposed first to unlabeled RNA, washed, and then exposed to labeled RNA. Machine background (20 count/min) has not been subtracted.

Unlabeled RNA added (μg)	Label bound to discs loaded with *E. coli* DNA (count/min)	Label bound to discs loaded with mouse DNA (count/min)
	Group 1	
0	30	154
50	30	96
100	33	86
200	28	65
400	26	63
600	23	58
	Group 2	
0	17	87
50	19	94
100	19	111
200	18	64
400	20	120
600	17	87
	Group 3	
0	32	456
400	24	295
800	17	205
	Group 4	
0	18	573
400	20	312
800	18	260

In order to wash off uncomplexed unlabeled RNA in the preincubation experiments, we simply swished the filters in a large beaker of 6XSSC at 67°C. The efficiency of this procedure in removing uncomplexed RNA was demonstrated in separate experiments with labeled RNA, where it was found that the uncomplexed RNA (ribonuclease-sensitive) carried over is of the same order of magnitude as that of the specifically bound RNA (ribonuclease-resistant), or less than 1 percent of the input RNA. At this level of carry-over, the amount of unlabeled RNA present during the hybridization with labeled RNA would be insufficient (less than 10 μg) to result in nonspecific depression of labeled hybrid formation.

We carried out a series of parallel experiments with T2 bacteriophage DNA and RNA from T2-infected *Escherichia coli* cells. In these experiments, the ratio of labeled RNA to DNA ranged from 0.044 to 92.5; in all cases competition was observed regardless of whether preincubation or simultaneous incubation was used. We did observe some quantitative differences in this series of experiments (*6*). It is pertinent to describe here one of these experiments, since it confirms the specificity of the action of the L-cell RNA in the experiments reported. We incubated 8.4 μg of labeled T2 RNA with discs containing 1 μg of T2 DNA, with and without 250 μg of unlabeled competing RNA. In the preincubation experiments, we observed a 30 percent decrease in label bound when the competing RNA was from T2-infected cells, and less than 1 percent decrease when L-cell RNA was used to compete. With simultaneous incubation, the corresponding decreases were 22 and 5 percent. The 5 percent decrease observed is well within the statistical variation we observe in simultaneous incubation experiments with mouse nucleic acids. In preincubation experiments, this variation is reduced to about 5 percent.

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References and Notes

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5. We immobilized DNA on nitrocellulose filters essentially by the method of Gillespie and Spiegelman [*J. Mol. Biol.* **12**, 129 (1965)]. Incubation was for 24 hours in 2 ml of 6XSSC (0.9*M* NaCl, 0.09*M* sodium citrate) (phage system) or for 16 hours in 1 ml of 6XSSC (mammalian system) at 67°C in the experiments where both RNA fractions were added simultaneously. Otherwise, the discs were pre-incubated for 24 hours with unlabeled RNA in 2 ml of 6XSSC, washed in 6XSSC, then incubated for an additional 24 hours with the labeled RNA in 2 ml of 6XSSC. The ribonuclease treatment and pre-ribonuclease wash were simplified by using a batch method, without filtration. After the ribonuclease treatment, the discs were washed with 50 ml of 6XSSC by filtration, the DNA-bearing side of the disc being turned downward. This method gave background values comparable with those obtained with the technique described by Gillespie and Spiegelman. Schleicher and Schuell 25-mm-type B7 filters were used; in the animal experiments, the prepared filters were cut into quarters before incubation with RNA. In the mammalian experiments, the "blank" filters carried 10 μg of *E. coli* DNA (*2*); in the T2 experiments they carried no DNA. Blank discs were subjected to the same preparative wash and vacuum drying as were the DNA-loaded filters; such discs have lower backgrounds than do untreated discs. RNA was prepared from L-cells pulsed for 45 minutes with ^{3}H-uridine and from *E. coli* cells pulsed with $^{32}PO_4$ or ^{3}H-uridine from 2 to 9 minutes after infection with T2 phage. All nucleic acids were prepared by repeated phenol extraction.
6. W. S. Riggsby, in preparation.
7. Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation. W.S.R. and V.R. are PHS postdoctoral research fellows.

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5 February 1968; revised 10 June 1968

Histochemical Localization of Renal Trehalase: Demonstration of a Tubular Site

Abstract. *Trehalase activity has been localized histochemically within the cortical tubules of the human, rabbit, and mouse kidneys. The specific localization of the enzyme supports the hypothesis that trehalase, in concert with a series of other enzymes which synthesize trehalose from glucose, functions in a mechanism for the reabsorption of glucose from the glomerular filtrate.*

Trehalase, which hydrolyzes the disaccharide trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) to two glucose moieties, is found in yeast, fungi, higher plants, and several phyla of invertebrate animals, especially insects (*1*). A disaccharidase specific for trehalose has been reported in rat intestine (*2*). The finding of prominent renal trehalase activity in numerous mammalian species without detectable blood trehalose has suggested a pathway for glucose reabsorption from the glomerular filtrate (*3*). The proposed mechanism involves hexokinase, phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase, trehalose-6-phosphate synthetase, trehalose-6-phosphate hydrophosphatase, and trehalase, with the overall reaction as follows (*4*):

$$2\ \text{glucose} + 2\ \text{ATP} + \text{UTP} \rightarrow$$
$$2\ \text{glucose} + 2\ \text{ADP} + \text{UDP} + \text{PP}_i + \text{P}_i$$

This hypothesis requires that trehalase be localized in the tubules of the renal cortex, the site of glucose resorption (*5*). We attempted to localize histochemically the enzyme in human, mouse, and rabbit kidneys. A human kidney was obtained from a 55-year-old white male during nephrectomy for a papillary carcinoma of the ureter. Kidneys were also obtained from a rabbit killed with pentobarbital and from a mouse killed with ether. The fresh kidney was cut into 3- to 4-mm slices containing cortex and medulla and either frozen on dry ice or fixed for 15 minutes in 2.5 percent gluteraldehyde in 0.1*M* phosphate buffer, *p*H 7.4, at 4°C followed by several changes of the phosphate buffer over 30 to 60 minutes. Sections (6 μm) cut in a cryostat were placed on coverslips coated with 0.2 percent gelatin, and incubated in a medium containing 1.8 mg of glucose oxidase, 4.5 g of Nitro Blue Tetrazolium, 2.7 mg of phenazine methosulfate, and 0.22 g of trehalose in 18 ml of 0.4*M* phosphate buffer, *p*H 6.0, at room temperature in the dark (*2*). Incubation times were from 5 to 40 minutes for the rabbit kidney, 3 hours for the mouse kidney, and 5½ hours for the human kidney. Control preparations consisted of the medium without trehalose or with sucrose substituted for trehalose.

The reduced formazan pigment, indicating the site of trehalase activity, was localized macroscopically in the cortex, prominently in the inner areas, of the mouse and human kidneys (Fig. 1). Direct enzymatic assay of the human kidney showed activities of 4.62 and 3.89 μmole of glucose produced per minute per gram of tissue (wet weight) for the inner and outer cortex, respectively (*3*). The entire cortex of the rabbit kidney was intensely stained. The larger distribution of the reduced formazan pigment in the rabbit cortex is indica-

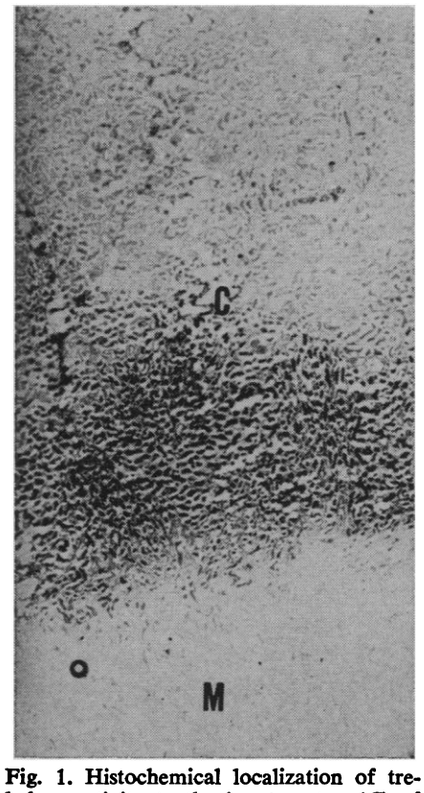

Fig. 1. Histochemical localization of trehalase activity to the inner cortex (*C*) of the human kidney. No medullary (*M*) activity is present (0.1 percent Kernechtrot counterstain, × 20).

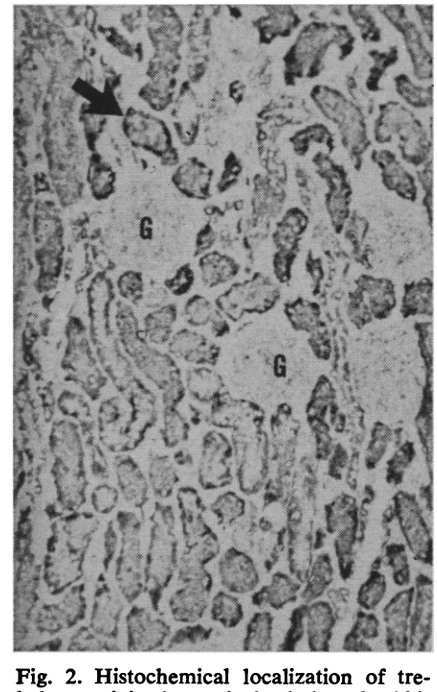

Fig. 2. Histochemical localization of trehalase activity in cortical tubules of rabbit kidney fixed in glutaraldehyde. Prominent proximal tubular staining is noted (arrow) as well as less intense staining in distal convoluted and collecting tubules. No glomerular (*G*) staining is noted (0.1 percent Kernechtrot counterstain, × 110). Control preparations showed no formazan deposition.