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COMPETITIVE HYBRIDIZATION WITH BRAIN RNA: FAILURE TO CONFIRM REPORTS OF NEW SPECIES OF RNA INDUCED WITH LEARNING

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There is considerable circumstantial evidence implicating protein and RNA synthesis in memory consolidation and increasing interest in work directed toward elucidating the macromolecular chemical processes underlying learning[1,2]. A derepression model has been advocated by Bonner[3], who also suggested a method by which the model might be tested. Newly induced RNA molecules would be gene products, present in the brains of learning animals but not naive animals, and they should be able to be pulse labeled and detected by competitive hybridization experiments.

The "detection of RNA species unique to a behavioral task" by competitive hybridization has now been reported by Machlus and Gaito[4,5,6]. When 50 μg of DNA was hybridized first with 50 μg of unlabeled, naive brain RNA and then with 50 μg of labeled, learned brain RNA, there was about 50% competition. But when the DNA was hybridized first with unlabeled, learned RNA and then labeled, naive RNA, no radioactivity was observed; there was apparently 100% competition. This was taken as evidence that there were new species of RNA in the brains of the trained rats. Our data show no differences between the RNA from trained and naive rats. Control experiments and consideration of other data in the literature for hybridization with nucleic acids from higher organisms lead us to believe that the competition values reported by Machlus and Gaito are unrealistic and incorrect.

We have attempted to reproduce their results as follows: Sprague-Dawley male rats, 200-250 grams, were injected intraventricularly with 40 μc 5-^{3}H-uridine in physiological saline or with physiological saline. Naive rats were returned to their home cages and sacrificed 90 min after injection. Trained rats were put into a training apparatus 60 min after

injection and allowed to become accustomed to the box for 15 min, and then were given 15 min of shock avoidance training, 15 trials in 15 min. They learned the task well within 4 or 5 trials. At the end of the training period the rats were sacrificed immediately, and total RNA was prepared from whole brain by a hot phenol procedure. DNA prepared by the procedure of Marmur[7] was denatured, immobilized on nitrocellulose membranes and hybridized in 1 ml of 6 X SSC at 66 degrees[8]. Fifty μg of DNA was hybridized first with 50 μg of unlabeled competitor RNA for 12 hr; then 50 μg of labeled testor RNA was added and the incubation was continued for 12 hr. Adding the testor RNA to the vial after pre-incubation of the competitor RNA with the DNA should maximize the observed competition.

Two experiments (Table 1) were performed under conditions comparable to those described by Machlus and Gaito, same amounts of DNA and RNA, same incorporation time for the labeled RNA, same temperature and time for annealing, etc. No statistically significant differences between trained and naive RNA were observed. All combinations of competitor and testor showed equal amounts of competition, about 36%. Small differences between trained and naive could go undetected, but these competition values are totally different from the 100% reported by Machlus and Gaito. Competitor RNA only partially blocked subsequent hybridization with testor RNA even when competitor and testor were identical, A and C.

A control experiment was performed in which 50 μg of DNA was hybridized for 12 hr with increasing amounts of 90 min pulse labeled brain RNA. There was a nearly linear increase in the amount of hybridized RNA in the range between 50 and 200 μg of RNA, which demonstrates that

50 μg of this RNA does not saturate its complementary gene sites on 50 μg of DNA under these conditions. Without saturation of complementary gene sites we could not expect complete competition. A competition curve with 50 μg of DNA, 50 μg of testor RNA and increasing amounts of competitor RNA showed only about 50% competition with 200 μg of competitor RNA. These observations are consistent with numerous others in the literature which show that very large excesses of short-term pulse labeled RNA from higher organisms are required to approach saturation of complementary DNA gene sites[9,10,11], and very large amounts of competitor RNA are required for effective competition[12,13]. Large amounts of competitor are especially important when the competition is accomplished by pre-incubation rather than simultaneous incubation.[14,15,16]

The reason for the lack of efficient competition with nucleic acids from higher organisms seems to be that many of the RNA species synthesized in the cell are present in such low concentrations that we cannot expect them to hybridize under the usual conditions[17]. Most of the hybridization normally observed is probably due to reassociation of RNA with redundant genes[18]. In presaturation competition experiments, unless we use very large amounts of RNA, we can expect only a small fraction of the messenger RNA species to hybridize during the first incubation. During the second incubation, most sites coding for messenger RNA will still be free, so little competition will be observed.

Competitive hybridization now has considerable popularity as a technique for demonstrating altered RNA metabolism in higher organisms, for instance during the course of embryonic development[19,20], during

liver regeneration[21,22], in malignant versus normal cells[23,24] and following hormone treatment[22,25,26,13]. Unfortunately, much of this type of work is difficult to interpret reliably. Several points should be kept in mind. Generally the hybridization of RNA from single copy genes is not clearly distinguished from that of RNA which is the product of repetitive genes. Some messenger RNA molecules may be coded for by repetitive genes, but most are not[27]. Hybridization in these systems is not stringently locus specific, and the amount of competition observed depends strongly on the ionic strength and the annealing temperature[12]. Much, if not most, of the RNA synthesized in the cell turns over very rapidly and never leaves the nucleus[28], and after a short-term pulse, much of the label will be in this type of RNA which is not functioning as messenger RNA. Slight changes in precursor pools can thus cause different populations of RNA species to be labeled, and could lead to competitive hybridization data which might erroneously be interpreted as demonstrating induction of new species of RNA. This last consideration is especially important for evaluating future hybridization results relating learning and brain RNA metabolism, as it appears that with the training situations which produce an increase in the incorporation of RNA precursors there are also ill-defined, regional, transient changes in precursor pool concentrations[29,30]. Nevertheless, the technique seems to be fairly sensitive, and under the proper conditions and with the proper controls it may well be capable of providing valuable information about brain function.

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Table 1. Hybridization Competition with Trained and Naive RNA

	Competitor RNA	Testor RNA	% Competition + S.D.
A	naive	naive	36.1 ± 3.1
B	naive	trained	32.7 ± 4.9
C	trained	trained	38.0 ± 2.3
D	naive	trained	35.8 ± 4.8

In the first experiment, A versus B, naive and trained RNA were compared as testor, and in the second experiment, C versus D, naive and trained RNA were compared as competitor. RNA was prepared separately from the brains of 4 rats for each type of RNA in each experiment. Each competition value was calculated from 4 independent determinations performed in duplicate. The specific activity of the labeled RNA was about 80 cpm/μg. Ribonuclease resistant hybrid RNA without competitor represented about 1.5% of the input label, or 60 cpm.

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