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Competitive DNA-RNA Hybridization of Microsomal and Nuclear RNA in Normal Tissues of the Rat[1]

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SUMMARY

Purified nuclear and microsomal RNA from rat brain, spleen, kidney, and liver were compared by means of competitive DNA-RNA hybridization. In the experiments described in this report a close similarity in the sequences detected by this technique is noted in the nuclear RNA of kidney, brain, and liver, while a portion of these sequences appear to be missing from the nuclear RNA of spleen. Microsomal RNA from each of the four tissues appears to be qualitatively different.

INTRODUCTION

Spleen, brain, liver, and kidney possess widely different functions *in vivo* which are reflected immunologically and enzymatically in the different proteins synthesized by the cells of these tissues. It is generally assumed that the molecular basis for this tissue differentiation lies in the transcription of different portions of the genome of the cell with their subsequent translation into protein. DNA-RNA hybridization studies, which directly measure homologies in RNA sequences suggest that the situation may be more complex. Not all sequences transcribed in the nucleus are detected in the cytoplasm (29). Furthermore, in tumors induced by 3'-methyl-4-dimethylaminoazobenzene, competitive DNA-RNA hybridization studies of nRNA indicated only quantitative differences in the sequences detected between the tumor and normal liver, whereas in the cytoplasm qualitative differences in the RNA sequences present were noted (30, 31). These studies and others in regenerating liver (7) indicate a possible control on transcription at the level of nuclear to cytoplasmic transport. Alternatively, the changes noted could be explained by differences in the stabilization of cytoplasmic mRNA templates (23).

Several studies with the competitive DNA-RNA hybridization technique have indicated differences in transcription of the sequences detected by this technique (8, 16, 33, 36) although this finding is not unanimous (18). In the present studies we have detected evidence of a close similarity in the nRNA sequences of kidney, brain, and liver, while a portion of these sequences appears to be missing from the nRNA of spleen. Microsomal RNA from each of the 4 tissues appears to be qualitatively different.

MATERIALS AND METHODS

The technique for RNA and DNA extraction and DNA-RNA hybridization has been covered in detail elsewhere (13) and only a brief outline is given below.

RNA Extraction. Brains, spleens, kidneys, and livers utilized for RNA extraction were taken from 350- to 600-g white male rats obtained from the Holtzman Co., Madison, Wis., which were starved overnight and then sacrificed between 8 a.m. and 10 a.m. the following morning. Animals from which radioactively labeled liver nRNA was obtained were given injections of 5 mCi of tritiated orotic acid and then killed 50 min or 3 hr later.

Organs were minced in ice cold 0.25 M sucrose, 0.05 M Tris(pH 7.6), 0.07 M KCl, 0.001 M homogenized in a Potter-Elvhjem homogenizer; and then fractionated into a post-mitochondrial supernatant and pellet containing nuclei, mitochondria, and lysosomes by centrifuging 10 min at 12,500 rpm with an S3-34 rotor in an RC-2B Sorval centrifuge. The supernatant was then recentrifuged at 28,000 rpm for 130 min with a No. 30 rotor in a Spinco L-65B ultracentrifuge, and the resulting pellet of microsomes was stored at −40° until use. Nuclei were isolated by suspending the 12,500-rpm pellet in 2.3 M sucrose containing 0.0033 M $CaCl_2$ and centrifuged at 28,000 rpm for 130 min.

The resulting cellular fractions were suspended in 0.01 M NaAc (pH 5.1), treated with SDS[4] at a final concentration of 0.5%, and then extracted 3 times with phenol at 60°. The pellets, interfaces, and phenol phases from these extractions were reextracted 3 times at 60° with $CHCl_3$ containing 1% isoamyl alcohol, and the aqueous fractions from both extractions were combined and precipitated with 2 volumes of 100% ethanol and 5 M NaCl added to a final concentration of 0.3 M NaCl with respect to the aqueous

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[4] The abbreviations used are: SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl: 0.015 M sodium citrate.

phase. The crude nucleic acids were then dissolved in double distilled H_2O centrifuged at 30,000 rpm for 30 min to remove bentonite and precipitated with 2 volumes of 100% alcohol, 0.3 M NaCl. The precipitates were subsequently treated with DNase and α-amylase, extracted with phenol and ether, eluted from a 20- × 1-cm column of Sephadex G-200 in 0.01 × SSC, and concentrated on an Amicon ultrafiltration apparatus. Filtration through G-200 removes all tRNA as judged by gel electrophoresis (13) and sucrose gradients (32).

DNA Extraction. DNA was extracted from nuclei of rat livers isolated as previously described (13). The nuclei were then suspended in 1 × SSC. This suspension was made to 2% SDS with 20% SDS; then 5 M NaCl was added to a concentration of 1 molar, the viscous solution was extracted 3 times with chloroform: 1-butanol (3:1) and overlaid with an equal volume of ethoxyethanol and the crude DNA spooled out. This DNA was dissolved by soaking in 0.1 × SSC, treated with pronase followed by phenol extraction to remove Pronase, and then dialyzed to remove the phenol. The DNA was treated with T1 and pancreatic RNase followed by bentonite treatment, a second Pronase treatment, and finally extraction with phenol. The aqueous layer was then overlaid with 100% ethanol and the purified DNA was spooled out and washed in 70 to 100% ethanol. It was dissolved in 0.01 × SSC and stored at 0–4° until use.

DNA-RNA Hybridization. DNA was immobilized on 47-mm Schleicher and Schuell B-6 filters in 6 × SSC then cut to small discs 8 mm in diameter. Each filter contained approximately 10 μg of DNA. Filters were heated to 80° in a vacuum oven prior to use. Groups of 4 filters were then incubated in 1 ml of 2 × SSC for 6 hr at 67°. They were then transferred to 0.2 ml of 2 × SSC containing 0.01 M *N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 7.1, and appropriate amounts of labeled and unlabeled RNA. Incubation at 67° was carried out for 20 hr after which filters were washed with 2 × SSC, incubated with T1 and pancreatic RNase and rewashed with 2 × SSC. Each filter was then dissolved in 0.25 M NaOH, neutralized with 0.15 M HCl, taken up with 10 ml of Scintisol, and counted in a Packard Tri-Carb scintillation counter. In 4 preliminary competitive DNA-RNA hybridization studies using labeled and unlabeled nuclear RNA and performed as were the studies to be presented below, standard errors of the mean duplicate samples for the 3 highest competition points averaged ±0.9% and did not exceed ±12% while at the highest competition point the average standard error was ±0.7% with a maximum of ±1.2%. With this degree of precision and the limited amount of nRNA available from other than liver tissue only single hybridizations were performed at each competition point with a difference between experimental points of 6% being significant at a minimum confidence interval of 0.95. Results with microsomal RNA as a competitor are supported by 2 or more studies. In all cases that could be checked wherein labeled RNA was present at concentrations at which rRNA would be saturating (20) the number of counts bound per filter was in significant excess of that which could be accounted for by rRNA alone (13). One to 3% of input counts were hybridized at 0 competitor concentrations in the present studies.

RESULTS

Competitive Hybridization of Nuclear RNA. RNA extracted from purified nuclei of liver, spleen, kidney, and brain was examined under conditions of temperature, ionic strength, and duration comparable to those of other investigators (8, 16, 18, 33, 36). Unlabeled nRNA from these 4 tissues was competed against 50-min pulse-labeled liver nRNA. The results are shown in Chart 1. Under these conditions, *i.e.*, 2 × SSC, 67° and 20-hr incubation no significant differences are noted in the competitive efficiency of unlabeled brain, kidney, or liver nRNA. Spleen nRNA is a less efficient competitor than the nRNA of the other tissues indicating that certain of the sequences in the liver nRNA which are being measured by this technique are absent from spleen nRNA.

In a second series of experiments kidney, spleen, and liver nRNA pulse-labeled with ^{3}H-orotic acid was competed with unlabeled liver nRNA to determine whether kidney or spleen nRNA contained sequences not present in liver nRNA. Fifty min were not a sufficient time to label spleen nRNA for hybridization studies so nRNA from tissues pulse-labeled for 3 hr was used. Fifty-min and 3-hr pulse-labeled liver nRNA have the same proportion of their radioactivity in the heterodispersed fraction although the specific activity of the heavier-molecular-weight species is greater with the shorter labeling time (38). As seen in Charts 2 and 3 both spleen and kidney nRNA appear to be competed to the same extent as liver nRNA by unlabeled liver nRNA.

These results show that spleen nRNA is lacking sequences present in the nRNA of the other 3 tissues. Under these conditions of specificity, however, the sequences which we are measuring in the nuclei of the other 3 tissues appear to be relatively similar. The possibility that brain nRNA might contain sequences which are absent from the nRNA of the other 3 tissues has not been excluded by these studies, however.

Competitive Hybridization of Microsomal RNA's. Microsomal RNA was used instead of total cytoplasmic RNA to minimize the possibility of spurious results from contamination by nRNA. Homogenates were made with 1 stroke with a loose-fitting Teflon pestle (7). Centrifugation of the homogenate at 12,500 rpm for 10 min removed all nuclei, all mitochondria, and most lysosomes when checked by electron microscopy (T. K. Shires and H. C. Pitot, unpublished observations). Postmicrosomal supernatants were carefully removed and discarded in an effort to minimize contamination of the microsomal pellet with any soluble species of nRNA which may be leaked by damaged nuclei. No DNA was detected by the diphenylamine assay in any microsomal RNA (or nRNA) preparations at high concentrations of RNA.

Microsomal RNA from liver, kidney, spleen, and brain were competed against 50-min pulse-labeled liver nRNA.

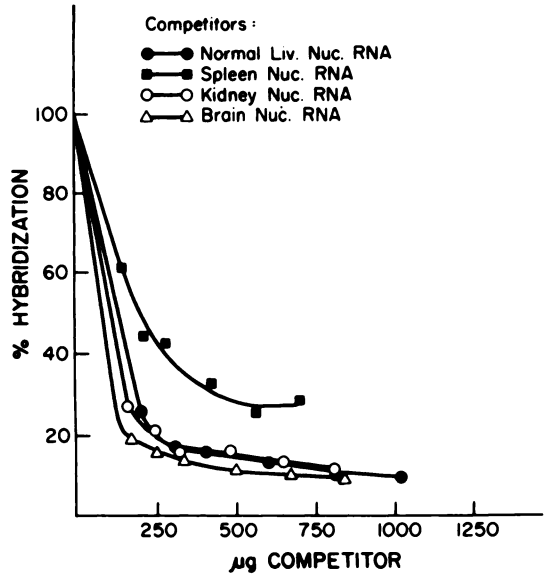

Chart 1. Comparison of nRNA from liver, kidney, spleen, and brain. ^{3}H-labeled normal RNA, 0.53μg, was incubated in 0.2 ml of 2 × SSC, 0.01 M *N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0) with four 8-mm diameter filters each containing approximately 10 μg of rat DNA in the presence of increasing amounts of unlabeled competing RNA. Specific activity was 92,000 cpm/μg. *Liv.*, liver; *Nuc.*, nuclear.

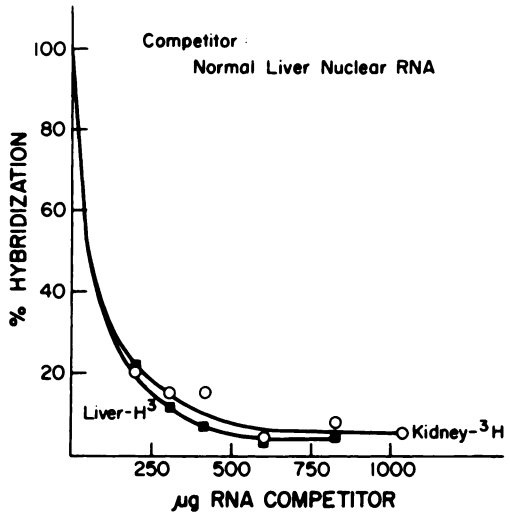

Chart. 2. Comparison of ^{3}H-labeled kidney (O) and ^{3}H-labeled normal liver nRNA (■). There was 0.81 μg of each of the ^{3}H-labeled nRNA's competing normal liver nRNA. For conditions see Chart 1. Specific activities were: kidney, 31,000 cpm/μg; and liver, 39,000 cpm/μg.

The results from 2 experiments are seen in Charts 4 and 5. The 2 highest competition points for liver microsomal RNA differed from kidney and spleen by confidence intervals of 0.97 or greater. Both spleen and kidney appear to lack sequences present in liver microsomal RNA. These findings using unlabeled kidney and liver microsomal RNA and labeled liver nRNA are supported by results from other studies in which the unlabeled competitors were competed against liver microsomal RNA labeled in the presence of 5-fluoroorotic acid for 3 hr, a situation in which only mRNA appears to be labeled. Kidney microsomal RNA appeared to lack sequences present in liver microsomal RNA (C. T. Garrett, unpublished observations).

The results of brain and liver microsomal RNA competition studies shown in Chart 5 indicate that populations of sequences confined to the nucleus in liver are transported to or stabilized in the cytoplasm of brain. Limited material permitted the use of only single competitor samples for brain microsomal RNA studies. A 2nd experiment again showed, however, a greater competitive efficiency of brain microsomal RNA than liver microsomal RNA for the sequences in liver nRNA.

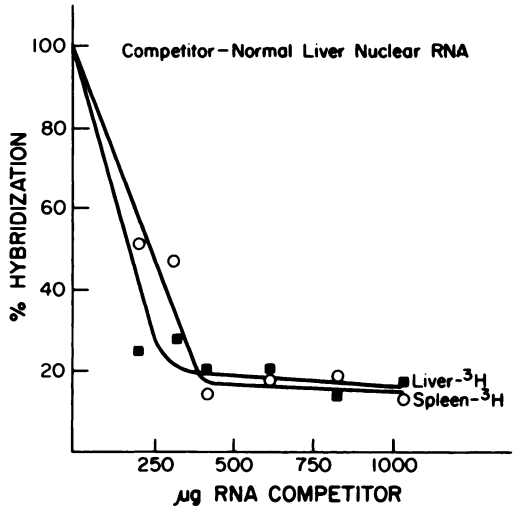

Chart 3. Comparison of ^{3}H-labeled spleen (O) and ^{3}H-labeled normal liver nRNA (■). Twenty-three μg of each of the ^{3}H-labeled nRNA's were incubated individually in the presence of increasing amounts of unlabeled competing normal liver nRNA. For conditions see Chart 1. Specific activities were: spleen, 600 cpm/μg; and liver, 39,000 cpm/μg.

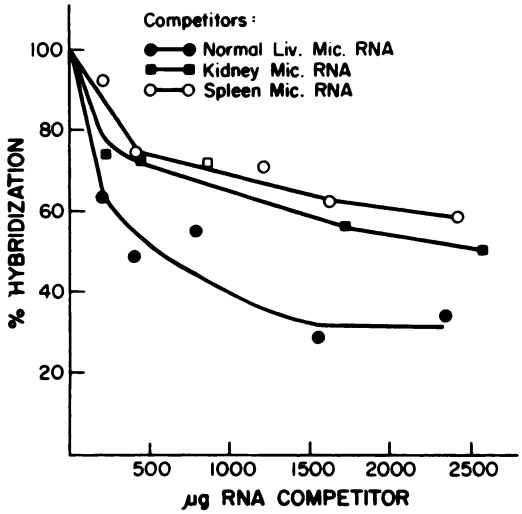

Chart 4. Comparison of spleen, kidney, and liver microsomal RNA. ^{3}H-labeled normal liver nRNA, 0.53 μg, was incubated in the presence of increasing amounts of unlabeled competing microsomal RNA. Conditions and specific activity are as in Chart 1. *Liv.*, liver; *Mic.*, microsomal.

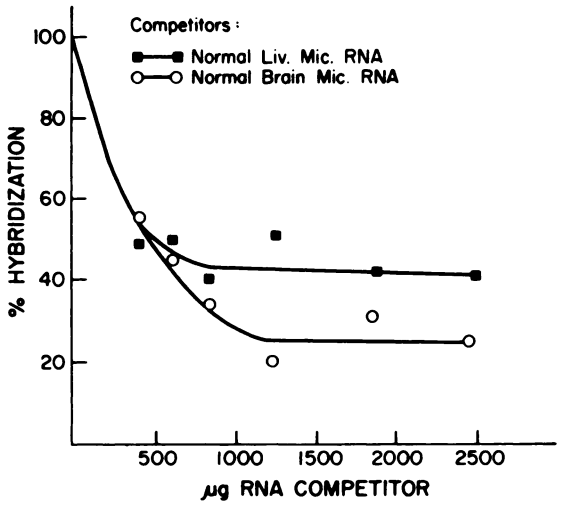

Chart. 5. Comparison of brain and liver microsomal RNA-^{3}H-labeled normal liver nRNA, 0.53 μg, was incubated in the presence of increasing amounts of unlabeled competing microsomal RNA. Conditions and specific activity are as in Chart 1. *Liv.*, liver, *Mic.*, microsomal.

DISCUSSION

The DNA-RNA hybridization technique as performed in these studies using pulse-labeled nRNA measures mainly sequences transcribed from highly reiterated parts of the eukaryotic genome (5, 6). Heterogeneous RNA sequences annealing in the 1st 2 to 6 hr appear to be transcribed from sequences reiterated 10,000 to 50,000 times (9, 21). rRNA cistrons number only 160 to 360 per haploid genome permitting a maximum annealing of only 4×10^{-4} to 5×10^{-4} μg of 18 S + 28 S RNA per μg of DNA (14, 19, 20, 34). It is for this reason that, unless RNA is pulse-labeled for very long periods of time, rRNA does not contribute significantly to the total counts annealed. For example, while total HeLa cell RNA pulse-labeled for 1 hr had a G + C content of 59%, the RNA which annealed to DNA using this labeled preparation had a G + C content of 46%, a value similar to DNA and heterogeneous RNA (26). In rat brain nRNA and microsomal RNA pulse-labeled for 15 to 16 hr, less than 5% of the counts annealed were ribosomal (35). The restricted amount of rRNA which may bind to DNA probably also accounts for the fact that purified rRNA saturates a given amount of DNA at 5% or less of the concentration of pulse-labeled RNA. Furthermore, the concentrations of unlabeled RNA required to achieve greater than 80% competition against labeled, purified rRNA is 5% or less that required for the same competitive efficiency against pulse-labeled RNA (1, 12, 14, 20, 24, 26, 27, 34, 35). The studies of Amaldi and Attardi (1) indicate that 34 μg/ml of 28 S RNA would be required to achieve 80% competition against labeled homologous 28 S RNA for 200 μg of DNA per ml. Even doubling this concentration to take into account the presence of 18 S RNA, this value is significantly below the 1250 μg/ml of unlabeled nRNA required to achieve 80% competition against RNA pulse-labeled for 50 or 180 min, as seen in these studies. nRNA extracted by the hot phenol method in this laboratory and examined by acrylamide gel electrophoresis indicates approximately 70% of a pulse label to be in heterodispersed RNA at both 60-min and 3-hr time points (38). Consideration of the conditions required for saturation of rRNA cistrons (12, 14, 20, 34) indicates that the concentration of 3-hr pulse-labeled RNA used in the study depicted in Chart 4 is in excess of that needed for saturation of these cistrons. Assuming that all of the specific activity were present in rRNA and that 4×10^{-4} μg rRNA annealed per μg DNA, the maximum number of counts hybridized in the absence of competitor using 3-hr pulse-labeled liver nRNA would be 624 cpm. In fact, 10,700 cpm actually hybridized, indicating that sequences other than those in rRNA account for most of the hybrid. If sequences unique to spleen and kidney are very rapidly turning over then the longer labeling time could result in a lower specific activity and hence difficulty in their detection (15). Assuming however, that liver also possessed similar unique rapidly turning over sequences qualitative differences would be expected between kidney and liver and brain and liver with 50-min pulse-labeled liver RNA which is not the case.

In the present studies considerable similarity appeared to be present between the sequences of liver, kidney, and brain nRNA which are detected by this technique. The possibility that at lower ionic strength and higher temperature some qualitative differences might become evident (17) is not completely excluded and will be the subject of subsequent studies.

The effect of using 3-hr pulse-labeled liver, kidney, and spleen nRNA instead of 50-min pulse-labeled RNA to examine for the production of unique species in kidney and spleen tissues probably has little or no significance.

The experiments with different competing microsomal RNA indicate that kidney and spleen lack sequences present in the microsomes of liver and that brain contains some sequences that the liver microsomal RNA lacks but which are present in normal liver nRNA. Additional studies using mixtures of the different microsomal RNA will be necessary to determine whether spleen or kidney contain sequences not present in liver microsomal RNA or liver contains sequences not present in brain microsomal RNA.

Most previous studies using competitive DNA-RNA hybridization which have indicated differences in RNA transcription have used total cellular RNA as a competitor (8, 33, 36). The present studies raise the possibility that those results may have been due to dilution of sequences not transported to the cytoplasm in the total RNA preparations. Quantitative differences of sufficient magnitude may appear as qualitative differences with standard competitive techniques (2, 4, 15). The finding by Sullivan (36) of qualitative differences between the nRNA of liver and thymus is in part similar to our finding with liver and spleen, the spleen also being rich in lymphocytes. Chromosomal RNA which may possess sequence differences between kidney and liver nRNA (16) may not have been detected in our studies with total nRNA either because of its extremely small concentration or low labeling due to a slow rate of turnover (3).

The precise role of reiterated sequences in DNA and

RNA is as yet unknown. Our finding of qualitative loss of sequences in spleen might in some manner relate to the high heterochromatin content (25) and hence large amount of nontranscribed DNA (25) in this tissue as compared to the other 3 tissues. The finding of considerably greater sequence variability in microsomal RNA than in nRNA would be consistent with a role for these sequences in the regulation of transport of mRNA from nucleus to cytoplasm or in mRNA template stabilization in the cytoplasm. This function is also suggested by the findings of sequences in nuclear informasomes not present in cytoplasmic RNA (22) and incorporation within or close association between reiterated sequences and polyadenylic acid-rich sequences (11). The addition of polyadenylic-rich sequences to certain mRNA appears to be an essential step for its transport to cytoplasm (10, 27).

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