DIFFERENCES IN RNA FROM RAT LIVER AND HEPATOMA REVEALED BY DNA-RNA HYBRIDIZATION

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SUMMARY

The population of RNA molecules from "minimal deviation" rat hepatoma (Morris hepatoma 5123 D) was compared by hybridization techniques with RNA molecules from normal rat liver. Total nuclear RNA from hepatoma seems to be poorer in some RNA classes as compared to normal liver. The cytoplasmic RNA from hepatoma exhibits greater competing potency when compared with cytoplasmic RNA from normal liver.

Rat hepatomas belonging to the well-defined group of "minimal deviation" tumors exhibit nearly normal karyotype, morphological similarity to normal rat liver, and grow slowly in inbred hosts. They show distinct differences in enzymatic pattern as compared to normal liver (Pitot, 1962; Potter, 1962). These features are heritable, indicating possible structural or functional alterations of the hepatoma cell genome. If this assumption is correct, we should expect differences in RNA classes between hepatoma and normal liver cells, which should be demonstrated by DNA-RNA hybridization techniques. In this communication we present a comparison of RNA from well-differentiated Morris hepatoma 5123 D with RNA from homologous liver cells of Buffalo rats.

EXPERIMENTAL

Animals. Male, healthy or hepatoma-bearing Buffalo rats from an inbred strain weighing 150 - 200 g were used. Hepatoma tissue was implanted intramuscularly and allowed to grow for about 6 weeks. To remove liver or hepatoma tissue, rats were sacrificed under light ether anaesthesia after 18 hours of fasting. For RNA labelling, carrier-free ^{32}P-labelled

phosphate was injected intraperitoneally (4 - 6 mCi) for 45 min or 3 hours before sacrificing the animal.

RNA extraction. Nuclei of hepatoma or liver cells were separated from cytoplasmic organelles by the method of DiGirolamo et al. (1964). Nuclear and cytoplasmic RNA were isolated by phenol extraction at pH 5.2 and 65° by the modified method of Scherrer and Darnell (1962). Bentonite (2 mg/ml) and polyvinylsulphate (2 µg/ml) were used throughout the procedure to depress RNase activity. Crude RNA preparations were digested with electrophoretically purified DNase (15 µg/ml) for 2 hours at 38°, followed by treatment for 6 hours at 38° with pronase at a concentration of 200 µg/ml. This step was followed by two phenol extractions, and macromolecular RNA was separated from "soluble" RNA by precipitation in 2 M NaCl. The traces of phenol in the macromolecular fraction were removed by exhaustive dialysis against 2 x SSC (SSC is 0.15 M NaCl and 0.015 M Na citrate). RNA concentration was calculated from the absorbancy at 260 mµ, assuming that a concentration of 1 µg/ml corresponds to an absorbancy of 0.02 for a 1 cm light path. Radioactivity was measured with a gas-flow, windowless counter for a time long enough to reduce the error to 5% or less.

DNA extraction. DNA was isolated from purified normal liver nuclei by the method of Savitsky (1966). Only this procedure gave DNA preparations which, after denaturation, were retained on nitrocellulose filters at a level approaching 100% of the input. Proteins were removed from DNA preparations by n-amyl alcohol : chloroform (1 : 10, v : v) treatment. Further purification was achieved by treatment with crystalline RNase (5 µg/ml) for 30 min at 38°. Before use, RNase was heated for 10 min at 90° and pH 5.0 to destroy DNase activity. Pronase (1000 µg/ml) was then added, and incubation continued for 6 hours at 38°. The preparation was again treated with n-amyl alcohol - chloroform, dissolved in 0.01 x SSC, and exhaustively dialysed against the same solution. DNA (conc. 100 µg/ml) was denatured by adjusting the pH to 12.7 with 1.0 N NaOH and kept at this pH for 15 min. The solution was then neutralized with 1.0 N HCl, heated at 100° for 10 min, and rapidly cooled.

Hybridization technique. Hybridization was performed, in 2 x SSC solution. The liquid method of hybridization (Gillespie and Spiegelman, 1965) was preferred to hybridization with DNA trapped on millipore filters for the following reasons: Considerable losses of mammalian DNA fixed on the filter during incubation in RNA solution occured. The rate of detachment of DNA was about 1 - 2% per hour of incubation in 2 x SSC at 65°. This rate could be reduced, but the loss was still high and difficult to control. The low concentrations of DNA used for hybridization in solution (5 - 10 µg/ml), and appreciable heterogeneity in base sequences of mammalian DNA, exclude possible errors caused by the reannealing effect of DNA (Kennell and Kotulas, 1968).

Ten to twenty µg of denatured DNA was mixed with 40-fold amounts of ^{32}P-RNA in a total volume of 2 ml of 2 x SSC, and incubated for 16 hours at 65° . ^{32}P-RNA-DNA complexes, after dilution with up to 50 ml 2 x SSC, were trapped on MF 50 nitrocellulose filters (Sartorius-Membranfilter, GmbH). The filters were thoroughly washed with 2 x SSC, uncomplexed RNA removed by RNase treatment (20 µg/ml, 1 hour, room temperature), and the radioactivity retained on the filters determined.

In competition experiments, excess unlabelled RNA was admixed to ^{32}P-RNA at levels indicated in the figures and this mixture was added to DNA for hybridization.

RESULTS AND DISCUSSION

In experiments not described here, it was established that the optimal incubation time for liquid hybridization at limiting DNA content was about 16 hours, and the optimal temperature 65°. Although the beginning of the plateau was observed after two hours, longer times were needed to secure full DNA saturation. The DNA to RNA ratio was chosen to be 40 as this value is on the saturation curve (Fig. 1). Hybrids formed under these conditions showed a high degree of specificity, as was demonstrated by experiments in which labelled rat liver RNA was complexed only in negligible amounts with bacterial (Streptococcus foecalis and Sarcina lutea) or calf thymus DNA.

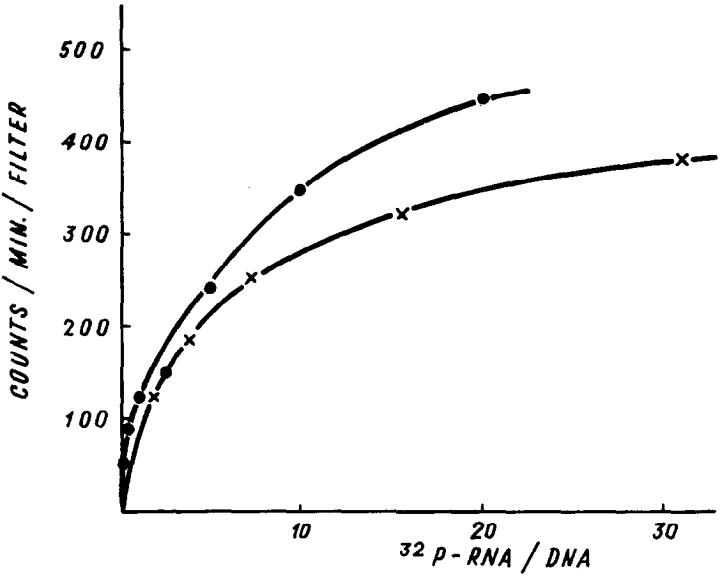

Fig. 1. Hybridization of total nuclear ^{32}P-RNA from normal rat liver with normal liver DNA at various RNA$_{32}$: DNA ratios. Curve ——•—— hybridization time 16 hrs.; ^{32}P-RNA isolated by phenol extraction at 65° after rejection of fraction isolated at 20° from normal rat liver, spec. activity 23 counts/min/ug . Curve ——×—— hybridization time 2 hours; ^{32}P-RNA isolated by Scherrer-Darnell hot-phenol technique from normal rat liver; spec. activity 248 counts/min/µg. Buffalo rats were injected with 6.5 mCi of ^{32}P per 150 g of body weight three hours before sacrificing.
Each point is the mean of triplicate samples.

Nucleotide sequence homology between total nuclear RNA from normal liver and normal liver DNA was found to be greater than that between total nuclear RNA from hepatoma and normal liver DNA. This is clearly demonstrated in competition experiments (Fig. 2). While a four-fold excess of unlabelled RNA from normal liver depressed hybrid formation to about 35% of the control, the nuclear RNA from hepatoma could compete only to the extent of 70%. Thus hepatoma nuclei seem to be deprived of some RNA classes as compared to normal rat liver. Similar conclusion were arrived at by Drews et al. (1968) and Chiarugi (1969). However, competing hepatoma RNA extracted from whole cells showed a higher nucleotide sequence homology with normal liver DNA than unlabelled RNA from whole

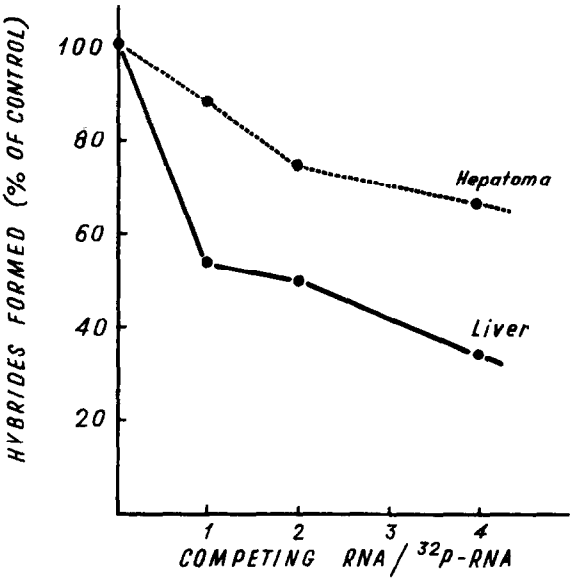

Fig. 2. Competition by unlabelled nuclear RNA from normal rat liver and hepatoma in hybrid formation between total nuclear ^{32}P-RNA from normal liver and normal liver DNA.

400 µg of ^{32}P-RNA (specific activity 154 counts/min/µg) were hybridized with 10 ug of DNA in the presence either of unlabelled nuclear RNA from normal liver (——•——) or of unlabelled nuclear RNA from hepatoma (----•----). The 100% level is 180 counts/min above the background.

Each point in this figure, and in Fig. 3 and 4, is the mean of triplicate samples. The maximal deviation from the mean was below 5%.

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normal liver cells (Fig. 3.). Similar competition curves were obtained with cytoplasmic RNA isolated from hepatoma and normal liver cells (Fig. 4). RNA isolated from hepatoma and normal liver cytoplasmic ribosomes did not show any differences in competition with normal ribosomal RNA. This was also found by Brimacombe and Kirby (1968) and Drews *et al.* (1968).

Thus it seems reasonable to conclude that the higher competing potency shown by hepatoma cytoplasmic RNA is not due to differences in ribosomal RNA in hepatoma as compared to normal liver cell. Since low-molecular fraction RNA, including sRNA, is discared during the isolation procedure, the observed higher competing effect of hepatoma cytoplasmic RNA with cytoplasmic normal liver RNA must be due to other RNA

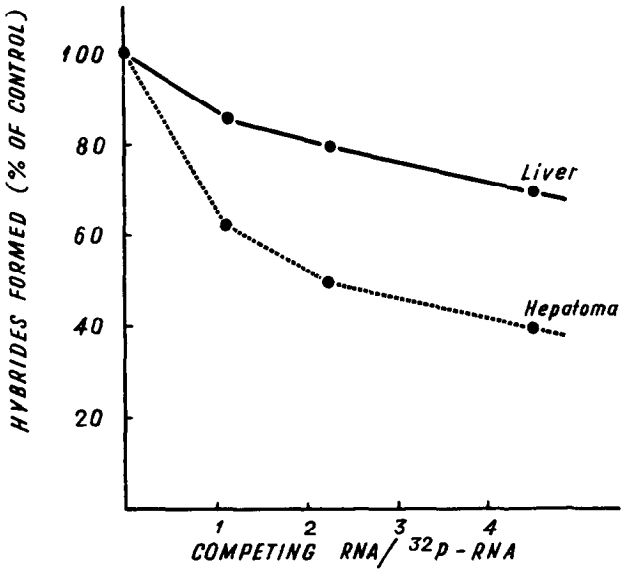

Fig. 3. Competition by unlabelled RNA from normal liver and hepatoma in hybrid formation between total nuclear ^{32}P-RNA from normal liver and normal liver DNA.
800 µg of ^{32}P-RNA (specific activity 264 counts/min/µg) were hybridized with 20 ug of DNA in the presence of total cell unlabelled RNA from normal liver (——•——) or in the presence of unlabelled RNA from hepatoma (----•----) . The 100% level is 86 counts/min above the backround.

classes than ribosomal RNA and sRNA.

When, in the competition reaction, total nuclear RNA from normal liver is compared with cytoplasmic RNA from normal liver, a greater dilution effect is observed with the former (cf. Figs. 2 and 3). At a four-fold excess of competing RNA over labelled RNA, the corresponding dilution was 45% for nuclear RNA and 70% for cytoplasmic RNA. This indicates that the considerable alteration of the population of RNA particles released from the nucleus to the cytoplasm in normal liver cell occurred. The postulated alteration could consist either of retention of some RNA classes within the nucleus, or conversion of considerable nucleotide sequences into free nucleotides by hydrolysis during RNA "maturation". Similar effects could be due to rearangement of certain regions in the RNA molecule or to elaboration of a new RNA class inside the cytoplasm.

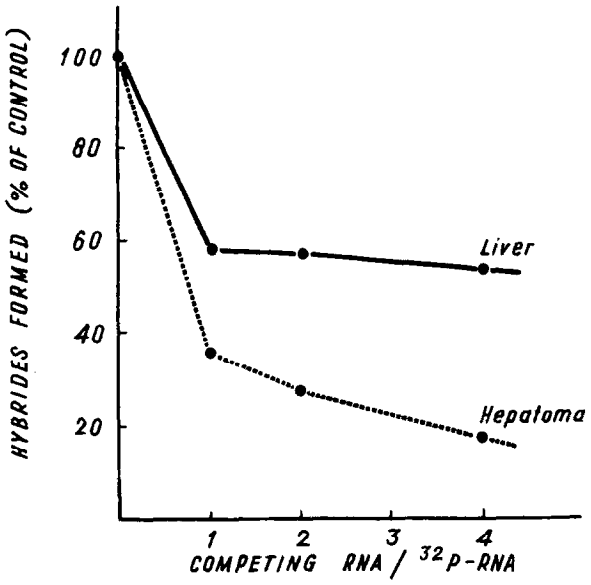

Fig. 4. Competition by unlabelled cytoplasmic RNA from normal liver and hepatoma in hybrid formation between total nuclear ^{32}P-RNA from normal liver and normal liver DNA.
800 µg of ^{32}P-RNA (specific activity 141 counts/min/µg) were hybridized with 20 µg of DNA in the presence of unlabelled cytoplasmic RNA extracted at pH 7.6 from normal liver (——•——) or from hepatoma (----•----). The 100% level of the control is 451 counts/min above the background.

The suggestion made by Drews et al. (1968) that in hepatoma cells the mechanism of RNA transfer between nucleus and cytoplasm is altered as compared to normal liver seems to be correct, but other possible explanations should also be taken into consideration.

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