# IRREVERSIBILITY OF THE ALTERATION IN RNA TRANSPORT INDUCED BY AN AZO-DYE CARCINOGEN IN THE RAT LIVER

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Received February 7,1974

## SUMMARY

Competitive RNA/DNA hybridization was used to study repair of the mammalian regulatory mechanism which selects only certain RNA species for transport to the cell cytoplasm. This mechanism, which is lost within a few days of feeding a liver carcinogen to rats, is not repaired eight months after returning the animals to a normal diet. Altered RNA transport is also demonstrated in the livers of adult rats whose parents were given the carcinogenic diet for three weeks ending at nine days gestation.

## INTRODUCTION

Specific RNA base sequences are restricted to the nucleus in normal rat liver (1,2,3) and other mammalian cells (4). Primary hepatomas induced by feeding a diet containing 3'meDAB (3'methyl-4-dimethylaminoazobenzene) or N-2-fluorenylacetamide have lost this control mechanism, at least for the transcripts of the repetitive fraction of DNA (2). This alteration occurs within a short time of treatment and correlates well with carcinogenicity of the nine drugs which have been tested on rat liver (8).

These experiments were designed first to determine the rate of repair of the RNA transport defect, and then to determine whether the lack of repair could be due to the continued presence of the carcinogen bound irreversibly within the altered cells.

## MATERIALS AND METHODS

Buffalo rats used were two months old at the start of carcinogen treatment. Normal liver was from rats four to five months old. Isotope labeled liver RNA was from a 6-week-old male rat sacrificed 100 minutes after intraperitoneal injection of 4 mc of (5-$^{3}$H) orotic acid, 26 Ci/mmole (Amersham-Searle, Arlington Heights, Ill.).

3'meDAB was obtained from Eastman Organic Chemicals, Rochester, N.Y., and was administered by feeding a low riboflavin diet containing 0.06% of the drug.

DNA was isolated from Morris hepatoma 5123, which has been shown to be indistinguishable from liver DNA in the filter hybridization assay (5). Hepatoma RNA was isolated from the third transplant of DB-2E, a parenchymal liver tumor induced in this laboratory by 3'meDAB (2).

Details of the isolation of nuclei and cytoplasm, purification of nucleic acids, and the hybridization-competition assay have been published (6). Purity of the cytoplasm and integrity of the nuclei were monitored by phase microscopy. Since the success of these experiments depends on the cytoplasm being absolutely free of nuclear contamination, homogenization was very gentle and no attempt was made to completely free the nuclei of cytoplasmic tags.

Hybridization incubations were done in 1.0 ml of 0.3 M NaCl at 68$^{o}$ for 18 hours in 3 ml screw-capped vials containing 6 mm DNA filters. Points plotted are averages of either two or three duplicate reactions. Background was determined by binding to Bacillus subtilis DNA and was less than 3 cpm above the machine background of 10 to 11 cpm. Each experimental point was counted a minimum of 100 minutes or 8000 counts.

## RESULTS AND DISCUSSION

Qualitative differences in RNA base sequences are demonstrated

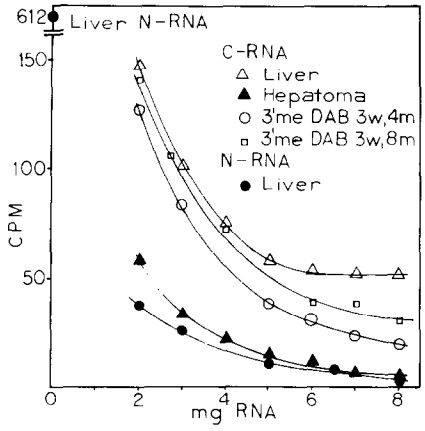


Figure 1. Effect of carcinogen on adult male rat liver. 9.2 µg of $^{3}$H-labeled normal liver nuclear RNA (specific activity 1740 cpm/µg) was allowed to react with 20 µg filter-bound DNA in the presence of unlabeled competing RNA: ○ and □ , livers of rats fed the carcinogenic diet for three weeks, followed by either 4 months or 8 months of Purina lab chow diet.

by differences in the end points of the curves of competition by two or more RNAs against a particular labeled RNA which, together with the DNA used, determines the range of genes assayed. The end point of a competition curve is either the level where it plateaus or the point at which it has reduced the binding of labeled RNA to less than twice background counts. Differences in the slopes of competition curves prior to the end point reflect quantitative differences in the component RNA sequences only and do not demonstrate qualitative differences.

Figure 1 shows the effect of the carcinogen 3'meDAB on the liver of the adult male rat. Normal liver cytoplasmic RNA lacks RNA species which are found in the nucleus of the same cells. Hepatoma cytoplasmic RNA competes completely, exhibiting the presence of transcripts of all of the gene families transcribed in normal liver nuclei. Livers of rats which have been fed a normal diet for many months after three weeks of carcinogenic diet do not have normal cytoplasmic RNA. The defect which allows RNAs which are normally restricted to the nucleus

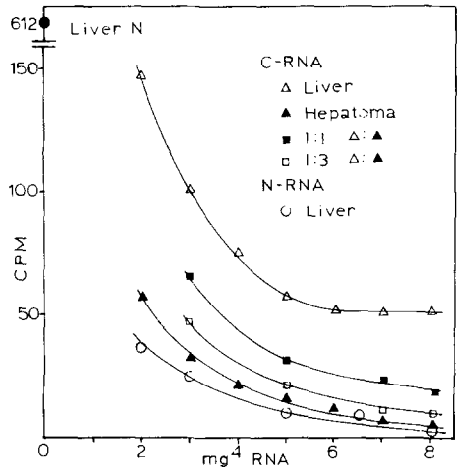


Figure 2. Determination of fraction of cells altered. Conditions of Figure 1. ■ and □ , mixtures of liver and hepatoma cytoplasmic RNAs in the proportions given.

to get into the cytoplasm has not been repaired in eight months.

The curves of carcinogen-treated liver in Figure 1 do not reach an end point within the solubility limit of the RNA, indicating a gross quantitative difference in competing RNA species between their cytoplasm and that of the hepatoma. This can best be explained as altered regulation of RNA transport in all cells of the hepatoma but in only a fraction of the cells of treated liver. The change demonstrated between four months and eight months after discontinuing carcinogen administration can be explained by replacement of damaged cells by division of normal cells during the very slow cell turnover characteristic of adult liver.

Figure 2 demonstrates the competition by artificial mixtures of liver and hepatoma cytoplasmic RNAs, for comparison with Figure 1. It appears that approximately 50% of the liver cells are still altered four months after resuming a normal diet, since the end point of this curve is similar to that of a mixture of equal amounts of liver and hepatoma cytoplasmic RNAs. The slopes are quite different, suggesting that the normal and altered cells in the treated liver may influence each other's rates of RNA synthesis or degradation.

The failure to repair the carcinogen-induced defect in RNA transport control suggests a permanent alteration of the genetic material. However, carcinogenic azo dyes are known to bind to subcellular components and remain bound weeks after they were administered (7), and this offers another possible explanation for failure to repair a defect which might operate at a lower level of control than the genes. To test the latter hypothesis, an experiment was designed to determine whether the carcinogen effect could be diluted out by cell proliferation.

Figure 3 shows that administration of the carcinogen during the first nine days of gestation, before the liver is formed, causes an alteration in RNA transport which persists in adult life. This suggests that the alteration involves a heritable change in the precursor cells for the liver and not simply an irreversible binding to a vital intracellular structure. However, the fact that the drug is retained for a long time in the adult (7) means that the embryos could have received small doses of the drug during the latter part of gestation also.

Figure 3 differs from Figure 1 in that cytoplasmic RNAs from the treated animals clearly plateau. This means that the rats treated prenatally have not lost the mechanism of selective transport, but they select more species of RNA for transport to their cytoplasm than do normal livers. Studies are in progress to determine whether the genes affected by prenatal carcinogen are active in normal prenatal liver or are activated at a later stage of development.

The treated rats of Figure 3 were littermates. Three males were sacrificed at 3 months of age and this experiment done. Two males and two females were sacrificed at four months of age and the experiment repeated, using the same preparations of labeled RNA and DNA. The three month and four month points are plotted separately

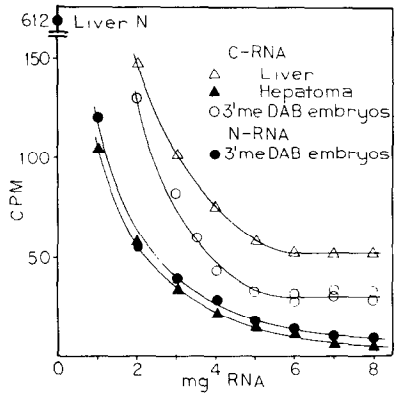


Figure 3. Effect of carcinogen on embryonic rat liver. Conditions of Figure 1. ○ and ● , three or four months old rats whose parents were fed the carcinogenic diet for three weeks ending at nine days gestation, followed by a diet of Purina lab chow through weaning.

in Figure 3, but all fall on a single line, demonstrating the high repeatability of this type of experiment.

Figure 3 also demonstrates that prenatal carcinogen does not prevent activation of transcription of genes which are transcribed in the normal adult liver; liver nuclear RNA from prenatally-treated rats competes completely.

These data indicate that a genetic control mechanism which operates at the translational level can be irreversibly altered by a chemical carcinogen, and the defect cannot be diluted by subsequent rapid proliferation of the cells.

ACKNOWLEDGMENT

This research was supported by Damon Runyon-Walter Winchell Cancer Fund grant DRG-1160 and by (U.S.) Public Health Service general research support grant 5 SOI RR05520.

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