Specificity of Chemical Modification of Ribonucleic Acid Transport by Liver Carcinogens in the Rat†

Ruth Whisler Shearer

ABSTRACT: Carcinogens and other drugs toxic to the rat liver were tested for their effect on the cellular mechanism which selects only a fraction of the RNA base sequences transcribed for transport to the cytoplasm. Cytoplasmic RNAs transcribed from families of related genes were compared by RNA–DNA hybridization in the presence of competing RNA. A single dose of any of five liver carcinogens (3′-methyl-4-dimethylaminoazobenzene, *N*-2-fluorenylacetamide, thioacetamide, aflatoxin B_1, dimethylnitrosamine) caused the appearance in the cytoplasm of RNAs which are normally restricted to the nucleus. The defect occurred in a detectable number of cells within 2 hr and was not repaired 2 months later. No such defect occurred in adult male rats after administration of ethanol, urethane, or α-naphthyl isothiocyanate, but dimethyl sulfoxide mimics the carcinogen effect.

Qualitative differences between RNA preparations can be demonstrated by the use of molecular hybridization techniques. When the hybridization of RNA to DNA is challenged with saturating amounts of competing RNA, differences in RNA populations transcribed from gene families can be detected. This assay was used to provide the first evidence that the RNAs which turn over in the nucleus of nonmalignant cells are qualitatively different from those which are transported to the cytoplasm (Shearer and McCarthy, 1967), and to characterize the nucleus-restricted RNA (Shearer and McCarthy, 1970a,b).

RNA base sequences that are restricted to the nucleus in normal rat liver are found in the cytoplasm in azo dye induced primary hepatomas (Shearer and Smuckler, 1972). Some of these RNAs are present in the cytoplasm of 19-day fetal liver and of liver regenerating 2.5 hr after 30% hepatectomy (Shearer and Smuckler, 1972). Abnormal migration of RNA out of the tumor cell nucleus is an early effect of the carcinogen, appearing in a significant fraction of the liver cells after the animals were fed a diet containing 0.06% 3′-methyl-4-dimethylaminoazobenzene for only 6 days (Shearer and (Smuckler, 1972). The fact that this defect can be detected so soon after administration of the azo dye facilitates comparison of this carcinogen with other carcinogens and noncarcinogenic drugs. The present study was designed to determine the correlation between carcinogenicity of a drug and its ability to alter the selection of RNA base sequences for transport to the cytoplasm.

Experimental Section

Male Buffalo rats (inbred from rats originally obtained from Simonsen Laboratories, Gilroy, Calif.) aged 3 months were used. Animals were fasted for 3 hr before receiving oral preparations by stomach tube at 1 hr past noon. Carcinogens used were 3′-meDAB[1] (Eastman Organic Chemicals, Rochester, N. Y.), *N*-2-fluorenylacetamide (Eastman), thioacetamide (Mann Research Laboratories, New York, N. Y.), dimethylnitrosamine (K & K Laboratories, Hollywood, Calif.), and aflatoxin B_1 (Calbiochem, Los Angeles, Calif.).

Other drugs tested were ethyl alcohol, dimethyl sulfoxide (Baker Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.), urethane (Sigma Chemical Co., St. Louis, Mo.), and α-naphthyl isothiocyanate (Eastman).

Isotope-labeled liver nuclear RNA was obtained from a 6-week-old male rat 100 min after intraperitoneal injection of 4 mCi of [5-^{3}H]orotic acid (26 Ci/mmol) (Amersham-Searle, Arlington Heights, Ill.).

DNA was isolated from Morris hepatoma 5123, which has been shown to be indistinguishable from liver DNA in the filter hybridization assay (Shearer, 1971).

The hepatoma RNA was isolated from the third transplant of DB-2E, a parenchymal liver tumor induced by chronic feeding of 3′-meDAB.

Details of the isolation of nuclei and cytoplasm, purification of nucleic acids, and the hybridization–competition assay have been published (Shearer and Smuckler, 1971, 1972). All homogenates used for cytoplasmic RNA met the following criteria. No distorted nuclei could be seen by phase microscopy, between 30 and 70% of the nuclei were free of attached cytoplasm, and no nuclei could be seen in the cytoplasm after centrifugation. An additional test for broken nuclei was a study of the reannealing characteristics of DNA isolated from liver and hepatoma cytoplasms which met the above criteria. This DNA was sheared, denatured, reannealed, and the resulting duplexes assayed for thermal stability. All duplexes melted in a single peak at high temperature, indicating no contamination of the mitochondrial DNA with nuclear DNA. If 3% of the nuclei were broken, the amount of nuclear repetitive DNA in the cytoplasm would have been approximately equal to the amount of mitochondrial DNA. A bimodal melting curve would have been found after overnight reannealing, since repetitive DNA contains related base sequences which cross-react to form imperfect duplexes which melt at a lower temperature. No trace of a low-melting peak was found, ruling out nuclear contamination (unpublished data).

RNA was purified by the hot phenol method (Shearer and McCarthy, 1967) and glycogen was removed by centrifuging at 35,000*g* for 40 min. DNA was purified by chloroform–

† From the Department of Molecular Biology, Pacific Northwest Research Foundation, Seattle, Washington 98104. *Received November 7, 1973.* This investigation 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.

[1] Abbreviation used is: 3′-meDAB, 3′-methyl-4-dimethylaminoazobenzene.

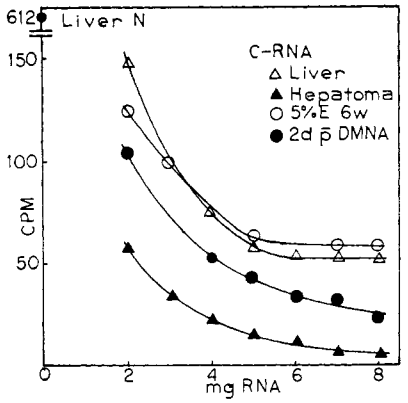

FIGURE 1: Comparison of cytoplasmic RNAs of hepatoma and normal and drug-treated liver. ^{3}H-labeled liver nuclear RNA (9.2 μg) (specific activity 1740 cpm/μg) was allowed to react with 20 μg of filter-bound hepatoma DNA in the presence of increasing amounts of unlabeled competing RNA. Incubation was in 1.0 ml of 0.3 N NaCl at 68° for 18 hr: (△) normal liver; (▲) hepatoma DB-2E (third transplant); (○) liver of rats given 5% ethanol in their drinking water for 6 weeks; (●) liver of rats given 0.2 μl of dimethylnitrosamine/100 g of body weight in olive oil by stomach tube 2 days prior to sacrifice.

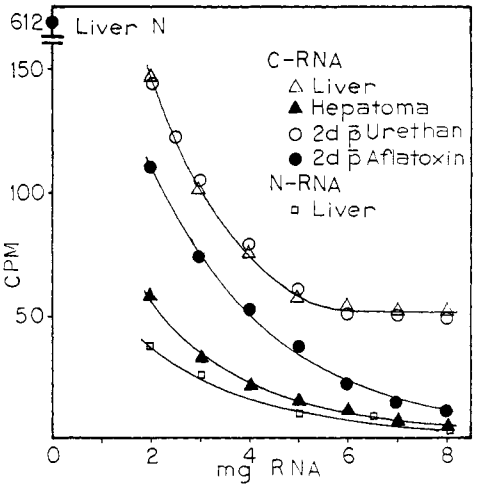

FIGURE 2: Conditions of Figure 1: (○) liver of rats given 100 mg of urethane/100 g in 0.15 N NaCl intraperitoneally 2 days prior to sacrifice; (●) liver of rats given 0.2 mg of aflatoxin B_1/100 g in olive oil intraperitoneally 2 days prior to sacrifice; (△,□) normal liver.

octanol extraction (Shearer and McCarthy, 1967). Purity was assayed by the spectrum of absorbance of ultraviolet light, as described previously (Shearer and Smuckler, 1971). DNA was heat denatured and applied to washed 25-mm membrane filters by very gentle suction, dried, and baked, and 6-mm disks were cut with a paper punch.

All hybridization incubations were in 1.0 ml of 0.3 M NaCl at 68° for 18 hr in 3-ml screw-capped vials. The ratios of nucleic acids used were determined by the two criteria which must be met in order to reach an end point, either plateau or complete competition, in order to detect qualitative differences between the competing RNAs. These are: (1) the amount of unlabeled RNA must be sufficient to saturate all of its gene families on the DNA which are large enough to be represented in the reaction of the labeled RNA without competitor, and (2) the ratio of competing RNA to labeled RNA must be high enough to dilute the labeled RNA to a negligible fraction of the total. The most efficient experimental design is one in which both ratios are optimum at the same level of competitor RNA, so that no component of the system is in great excess over what is needed.

Points plotted are averages of either two or three duplicate reactions. Duplicate points are not plotted separately because on the significant part of the curves, $\geq$5 mg of RNA, duplicates never varied by more than 2.1 cpm. On the initial slopes, 1–4 mg of RNA, maximum variation was 6.7 cpm. The data are plotted on a broken scale to emphasize the significance of the end points and the irrelevance of the points on the slope of the curves. Background was determined by binding to *Bacillus subtilis* DNA and was negligible (less than 3 cpm above the machine background of 10–11 cpm). Each experimental point was counted a minimum of 8000 counts or 100 min in a Beckman LS-100 scintillation counter using polyethylene minivials and toluene–2,5-bis-2-(5-*tert*-butylbenzoxazolyl)thiophene (Beckman).

Figures 1, 2, and 5 are simultaneous experiments plotted separately for clarity, as are Figures 4 and 5. The control, dilution of the labeled RNA with identical unlabeled RNA, is included in Figure 2.

Results

Ubiquity of Loss of the Selective RNA Transport Mechanism after a Single Dose of Carcinogen. Rats given a single oral dose of the liver carcinogens thioacetamide, 3′-methyl-4-dimethylaminoazobenzene, *N*-2-fluorenylacetamide, dimethylnitrosamine, or aflatoxin B_1 show a loss of selection in the transport of RNA to the cytoplasm after 2 hr, 2 days, and 2 months. The dose given equals a single daily dose used in tumor induction by chronic feeding, with the exception of the thioacetamide experiment in which ten times the daily feeding dose was given.

Representative curves from these experiments are shown in Figures 1–4. The maximum RNA:DNA ratio used is 400, and the maximum RNA:labeled RNA ratios are 870 in Figures 1 and 2, and 400 in Figures 3 and 4. The normal liver and hepatoma competitions are complete with 6 mg of unlabeled RNA, where the ratios are $^3/_4$ of the maximums used. Some of the carcinogen curves do not reach complete competition, but all fall well below the plateau of normal liver cytoplasmic RNA, indicating that carcinogen-treated cytoplasms contain RNA species which are not found in the cytoplasm of normal liver. After the single dose of the nitrosamine or the azo dye, liver cytoplasmic RNA does not have a high enough concentration of competing sequences to dilute the label. Continuing this type of experiment beyond 8 mg/ml is unsatisfactory because of solubility problems.

Differences in the slopes of the curves result from differences in the fraction of cells altered, not in the fraction of genes affected. The latter would result in curves which plateau at different levels below the normal; the curves for carcinogen-treated liver shown do not plateau. The fraction of cells altered by a single dose of carcinogen is much less than in the hepatoma. Differences between the curves for carcinogen-treated RNAs and the hepatoma RNA do not reflect qualitative differences, since none of these curves plateau. Differences in slope indicate quantitative differences in the component RNA species only.

Persistence of Altered RNA Transport after a Single Dose of Carcinogen. The competition curves for liver cytoplasmic RNA two months after a single dose of 3′-methyl-4-dimethylaminoazobenzene or *N*-2-fluorenylacetamide indicate a lack of repair of the transport defect during this time (Figures 3 and 4). There is a change in the slope of the curve after azo dye which

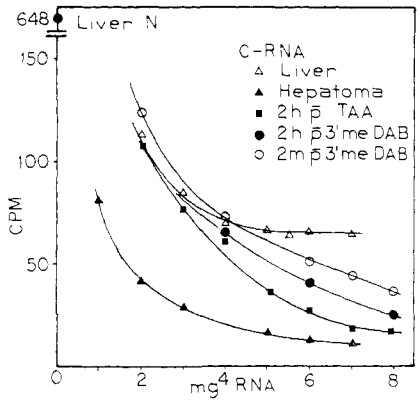

FIGURE 3: Conditions of Figure 1 except 20 μg of labeled RNA (752 cpm/μg) was used: (■) liver of rats given 20 mg of thioacetamide/100 g in olive oil by stomach tube 2 hr before sacrifice; (●,○) livers of rats given 3 mg of 3'-meDAB/100 g in olive oil by stomach tube 2 hr (●) or 2 months (○) before sacrifice.

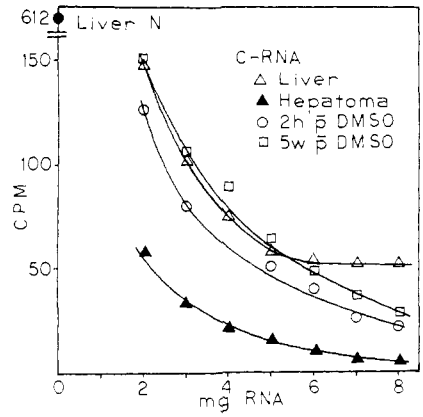

FIGURE 5: Conditions of Figure 1: (○,□) livers of rats given 0.1 ml of dimethyl sulfoxide/100 g in olive oil by stomach tube 2 hr (○) or 5 weeks (□) before sacrifice.

could result from death of some of the affected cells and replacement by division of normal cells.

Preservation of the Selective RNA Transport Mechanism after Treatment with Drugs Which Are Not Carcinogenic in Adult Rat Liver. Rats given 5% ethanol in their drinking water for 6 weeks show no abnormality in their liver cytoplasmic RNA by this assay (Figure 1). The same is true for adult rats given a single intraperitoneal dose of urethane (Figure 2), although this drug is carcinogenic for various organs when given to newborn rats.

The very toxic drug α-naphthyl isothiocyanate presents a somewhat different picture (Figure 4). Two days after treatment, there is a marked quantitative change in the liver cytoplasmic RNA which obscures the end point of the curve by putting it beyond the solubility limit of the RNA. However, two months later the cytoplasmic RNA is indistinguishable from that of normal liver, as was the histology. The dose used was lethal to 80% of the rats four days after treatment.

Dimethyl Sulfoxide, an Exception to the Pattern. In early experiments, dimethyl sulfoxide was used as a solvent for aflatoxin B_1, but the control experiments using dimethyl sulfoxide alone showed the same pattern of altered RNA transport as those using the carcinogen. Long-term administration of Me_2SO resulted in alteration of enough liver cells that the liver cytoplasm became able to compete completely. Figure 5 demonstrates that this alteration in rat liver persists after a single dose.

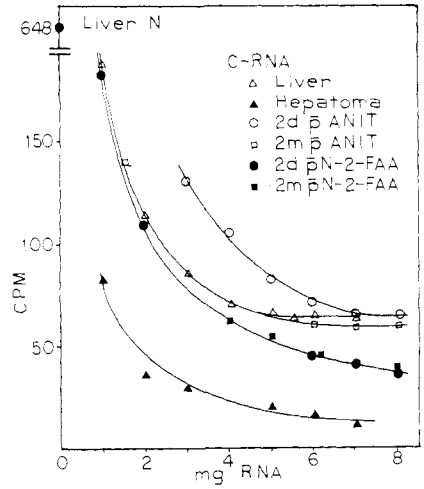

FIGURE 4: Conditions of Figure 3: (○,□) livers of rats given 10 mg of α-naphthyl isothiocycanate/100 g in olive oil by stomach tube 2 days (○) or 2 months (□) before sacrifice; (●,■) livers of rats given 1.5 mg of *N*-2-fluorenylacetamide/100 g in olive oil by stomach tube 2 days (●) or 2 months (■) before sacrifice.

Discussion

These studies on the early effects of liver carcinogens on RNAs transcribed from families of related base sequences continue to indicate the significance of gene regulation at the level of transport of RNA from the cell nucleus to the cytoplasm. The early and irreversible loss of transport control in carcinogen-treated liver correlates well with carcinogenicity.

Dimethyl sulfoxide has not been shown to be carcinogenic, but it is mutagenic in yeast (Yee *et al.*, 1972), and teratogenic in chick embryos (Mawhinney and Schenken, 1972). It has been implicated in altering the state of differentiation of neuroblastoma cells (Epstein, 1972; Furmanski and Lubin, 1972) and murine erythraleukemia cells (Friend *et al.*, 1971), and in increasing the production of C-type RNA virus in cultured tumor cells which have been pretreated with iododeoxyuridine (Stewart *et al.*, 1972) or bromodeoxyuridine (Weinstein *et al.*, 1972). The data presented here are one more confirmation that dimethyl sulfoxide is not simply an innocuous solvent.

Selective RNA transport is one form of translational control, and has been shown to be involved in the difference between normal and regenerating liver (Church and McCarthy, 1967), and between normal and estrogen-stimulated uterus (Church and McCarthy, 1970). Nothing is known about the mechanism involved in the normal cells, so its alteration in tumors could involve changes in the structure of the nuclear membrane, changes in processing of the giant nuclear RNA, changes in specificity of the proteins which bind to RNA, or changes in other undiscovered factors.

Whether or not the RNAs abnormally found in the cytoplasm in carcinogen-treated liver are translated into protein remains to be determined. However, many new proteins as well as reactivated fetal proteins have been demonstrated in carcinogen-treated liver, suggesting translation of additional RNAs not found at the sites of protein synthesis in normal liver.

The functional relationships between single-copy genes and families of related genes in mammalian cells have not been determined. However, there is growing evidence that they are closely interspersed in the chromosomes (Grouse *et al.*, 1972) and that transcripts of both kinds of gene can be found on the same polysomes (Dina *et al.*, 1973).

The translation of RNA sequences which do not normally leave the nucleus at any stage of development would produce antigens for which the animal has no immunological tolerance, resulting in cells highly antigenic to the host. Chemically induced tumors are noted for their extreme antigenicity.

The transformation of a normal cell to a malignant one probably requires two (Epstein, 1972; Knudson and Strong, 1972) or more (P. R., *Nature*, 1973) basic alterations at the molecular level. If any one of the required changes could be prevented, no cancer would occur. For this reason it is imperative to define the parameters affecting susceptibility to any aspect of altered control suspected of being a basic step in carcinogenesis, even though it is obviously not the only alteration required. The loss of the control mechanism which selects only certain RNAs for transport out of the nucleus appears to be such a relevant defect. However, it seems intuitively that only a few of the RNAs affected might be necessary to establish the malignant state—those which are translated into proteins needed for cell division, those which code for proteins which alter the cell surface so that it does not respond to contact with other cells and can therefore metasthesize, and possibly the products of mutated genes or viral genes which the cell would not otherwise select for transport to the cytoplasm.

When all of the basic steps in malignant transformation have been defined, it will be possible to test chemicals for their ability to induce any one step, and hitherto undetected cocarcinogens will be identified. Dimethyl sulfoxide may be one of these, affecting only RNA transport and lacking other attributes of a complete carcinogen.

An even more important result of defining the stages of carcinogenesis in molecular terms will be the ability to assay potentially therapeutic drugs for their ability to revert any one of the basic steps of transformation and therefore stop tumor growth.

Acknowledgment

I thank Dr. Edward A. Barker for the thioacetamide-treated rats.

References

Church, R. B., and McCarthy, B. J. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1548.

Church, R. B., and McCarthy, B. J. (1970), *Biochim. Biophys. Acta 199*, 103.

Dina, D., Crippa, M., and Beccari, E. (1973), *Nature (London), New Biol. 242*, 101.

Epstein, J. H. (1972), *Cancer Res. 32*, 2625.

Friend, C., Scher, W., Holland, J. G., and Sato, T. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 378.

Furmanski, P., and Lubin, M. (1972), *J. Nat. Cancer Inst. 48*, 1355.

Grouse, L., Chilton, M.-D., and McCarthy, B. J. (1972), *Biochemistry 11*, 798.

Knudson, A. G., Jr., and Strong, L. C. (1972), *J. Nat. Cancer Inst. 48*, 313.

Mawhinney, B. S., and Schenken, L. L. (1972), *Nature (London), New Biol. 236*, 155.

P. R. (editorial) (1973), *Nature (London) 241*, 238.

Shearer, R. W. (1971), *Biochem. Biophys. Res. Commun. 43*, 1324.

Shearer, R. W., and McCarthy, B. J. (1967), *Biochemistry 6*, 283.

Shearer, R. W., and McCarthy, B. J. (1970a), *J. Cellular Physiol. 75*, 97.

Shearer, R. W., and McCarthy, B. J. (1970b), *Biochem. Genet. 4*, 395.

Shearer, R. W., and Smuckler, E. A. (1971), *Cancer Res. 31*, 2104.

Shearer, R. W., and Smuckler, E. A. (1972), *Cancer Res. 32*, 339.

Stewart, S. E., Kasnic, G., Jr., Draycott, C., and Ben, T. (1972), *Science 175*, 198.

Weinstein, I. B., Gebert, R., Stadler, U. C., Orenstein, J. M., and Axel, R. (1972), *Science 178*, 1098.

Yee, B., Tsuyumu, S., and Adams, B. G. (1972), *Biochem. Biophys. Res. Commun. 49*, 1336.