A Search for Gene Derepression in RNA of Primary Rat Hepatomas

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SUMMARY

The technique of RNA/DNA hybridization in the presence of competing RNA was used to look for genetic derepression in primary rat hepatomas. No qualitative difference was demonstrable between the RNA's of different hepatomas and normal liver, although quantitative differences related to frequencies of various cell types do exist. The results preclude any gross activation of gene families during tumorigenesis but do not assay for inactivation. The data suggest that an alteration in transport of RNA out of the cell nucleus may be important.

INTRODUCTION

The biochemical basis for malignant transformation is not well known. Significant alterations in RNA metabolism have been reported in a variety of chemically induced liver cancers and even following a single exposure to certain carcinogens or their analogs (22). Derepression of genes with resultant transcription of new mRNA has been postulated to explain the unusual genetic activity in tumors (9, 11, 13, 15). Attempts have been made to demonstrate this in transplantable tumors (3, 14, 20), but only in the case of the mouse Taper liver tumor (3) were the results complete enough to support the conclusion that qualitative differences exist. This tumor has been transplanted over 100 times and probably has undergone a number of alterations related to passage and to cell selection. Observed differences from normal liver may not necessarily be related to the original malignant transformation.

This study was undertaken to extend these results to primary tumors. The experimental design utilized chemically induced liver tumors which were assessed morphologically as well as chemically. The formation of hybrid RNA/DNA molecules by incubation of isotopically labeled RNA with denatured DNA can be decreased by the presence of large amounts of unlabeled RNA if it contains similar base sequences which compete for the same sites of complementarity on the DNA. Properly designed experiments, with RNA of high specific activity, can detect qualitative as well as quantitative differences between RNA's from different sources.

MATERIALS AND METHODS

Liver tumors DS-1A, DS-2A, DS-9A, and DS-10A were induced in male Sprague-Dawley rats by continuous feeding of a low-riboflavin diet (Arcasoy No. 64707, Nutritional Biochemicals Corporation, Cleveland, Ohio) containing 0.06% 3'-methylidimethylaminoazobenzene for 4 months. Rats carrying Tumors DS-1A and DS-2A were fed a normal diet for the last month before sacrifice. Tumor FS-1A was also induced in a Sprague-Dawley rat following feeding of ground Wayne Lab-Blox (Allied Mills, Inc., Chicago, Ill.) containing 0.03% N-2-fluorenylacetamide for 9 months. Morris hepatoma 5123 was obtained from Dr. Robert Wissler, University of Chicago, Chicago, Ill., and maintained by serial transplant in the thighs of Buffalo rats. Novikoff hepatoma was obtained from Dr. John Holcenberg, Department of Medicine, University of Washington, and was transplanted in the thighs of Sprague-Dawley rats. For assessment of the effect of the azo dye acting as a toxin, Sprague-Dawley rats were fed a low-riboflavin diet containing 0.06% AB3 for 3 months ("toxic liver").

Animals were sacrificed by exsanguination under light ether anesthesia. Livers were removed and chilled immediately, and tumors were isolated by gross dissection. Fragments of the host liver and tumors for histological examination were fixed in neutral, buffered 10% formalin. Paraffin-embedded tissue was sectioned at 5 μ and stained with hematoxylin and eosin. Tissue for ultrastructural study was fixed in 2% osmium tetroxide, dehydrated in alcohols, and embedded in Epon. Thin sections were cut with diamond knives and stained with uranyl acetate and lead hydroxide. The resulting specimens were examined in an RCA-3G electron microscope.

Nuclear and cytoplasmic fractions were separated following gentle homogenization of the tissue in 0.015 M NaCl solution containing 0.1% Tween 80 (polyoxyethylene sorbitan monoooleate). The homogenate was centrifuged at 200 X g for 10 min, followed by washing of the nuclear pellet in 0.15 M NaCl solution and repeated centrifugation of the cytoplasm at 500 X g until no nuclei could be detected microscopically. No attempt was made to obtain completely clean nuclei; the experiments demand clean cytoplasm, free of any broken nuclei. However, over 50% of the nuclei were always free of...
cytoplasmic tags, ensuring that no species of cytoplasmic RNA would be deleted from the cytoplasmic fraction by its adherence to membranes attached to the nuclei.

Isotope labeling of tumor cell RNA was done in monolayer cell culture. Tumors were disrupted by passing through a 60 mesh screen, and the cells were cultured in Eagle's medium with nonessential amino acids, 4 mM glutamine, and 15% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). Tumor DS-10A was labeled after 2 passages in culture, and Morris hepatoma 5123 was labeled after 16 days in primary culture, each with 3H-labeled uridine (>20 Ci/mmmole, New England Nuclear, Boston, Mass.) 20 µCi/10⁶ cells for 1 hr in fresh medium.

The isolation of RNA and DNA and binding of DNA to membrane filters have been described (16–18). DNA was isolated by hot phenol extraction in an acidic solution of 0.5% sodium dodecyl sulfate in 0.2 M LiCl. DNA was isolated by chloroform-octanol extraction in 1% sodium dodecyl sulfate.

RNA was used when the ratio of its absorbance at 260 µm to that at 280 µm was >2.0, and its A₃₂₀/A₂₆₀ ratio was >2.3. DNA was purified until its A₂₆₀/A₂₈₀ was >1.90 and A₂₆₀/A₇₅₀ > 3.0. DNA was heat denatured and applied to washed 25-mm membrane filters by very gentle suction. Six-mm discs were cut with a paper punch.

All hybridization incubations were in 0.5 ml of 0.3 M NaCl solution at 67°C for 17 hr. RNA's were in solution, with a single DNA filter disc in each reaction vial. Background was determined by binding to Escherichia coli DNA and was negligible (less than 2 cpm) at high levels of competing RNA from Novikoff hepatoma. Each experimental point was counted a minimum of 8000 counts or 100 min in a Packard Tri-Carb scintillation counter.

RESULTS

Histological Examination. Histological examination of the tumors formed following azo dye feeding revealed that they were comprised of 2 cell types, those resembling parenchymal cells and those bordering ductlike channels. Tumor DS-2A showed very few ducts. DS-1A had more ductal cells than DS-10A, and FS-1A was the most ductal primary tumor in this study.

Ultrastructure of these tumors was very heterogeneous, even within a single grid square. Parenchymal and ductal areas were intermingled and were often separated only by the basal lamina of the ductal cells and a single layer of fibroblasts, blood cell precursors, or undifferentiated tumor cells. All tumors contained large areas of undifferentiated cells (Figs. 1 and 2). All cells observed in mitosis lacked cytoplasmic differentiation. Other cells were caricatures of differentiated cells; parenchymal cells often had excessive amounts of stacked rough endoplasmic reticulum (Fig. 1) as well as microbodies and fat droplets. Ductal cells had very many microfibrils and exaggerated interdigitation of their cell membranes (Fig. 3). This is in contrast to host liver, in which parenchymal cells were deficient in rough endoplasmic reticulum, as reported previously (1), but bile ducts appeared normal.

Comparison of Primary Tumors. Tumor DS-10A was grown in culture, labeled as described, and its RNA was hybridized with rat liver DNA. RNA's derived from Tumors DS-1A, DS-2A, DS-10A, normal liver, and liver from AB-fed animals were used for competition experiments. Chart 1 shows that Tumors DS-1A and DS-2A are able to compete just as completely as control Tumor DS-10A, indicating that all gene families active in DS-10A are also active in the other tumors. The slight difference in slope of these curves suggests differences in the frequencies of the various RNA components. These differences seem related to the ratio of parenchymal to ductal areas seen histologically.

Chart 2 shows that RNA from a more ductal primary tumor (FS-1A) induced by a different carcinogen also contains all families of RNA present in the 3'-methylidimethylaminoazobenzene-induced tumors.

Comparison of Liver with Primary Tumor. As a test for differences in gene expression between normal liver and induced liver tumor, a series of competition experiments between DS-10A RNA and RNA from liver was undertaken. Chart 1 illustrates the competition of RNA's obtained from normal and AB-treated liver against labeled primary tumor RNA. The slopes suggest quantitative differences, and the liver RNA competition curves appear to be approaching a plateau. However, these curves are incomplete. No end point has been reached, so no conclusion can be drawn about qualitative differences between tumor and normal RNA's. Competitive hybridization is not complete unless each experimental curve either reaches a plateau (defined by at least 3 experimental points on a line parallel to the abscissa) or drops to an insignificant value (less than double the counterbackground). Differences in the slopes of the curves prior to the end point indicate quantitative imbalances among the various component species of RNA but do not relate to the question of qualitative differences. Since qualitative differences in RNA base
sequences present are indicated by differences in the end point levels, both control and experimental curves are needed in order to justify conclusions. Two criteria must be met in order to reach an end point, either plateau or complete competition. The amount of RNA must be sufficient to saturate all of its complementary sites on the DNA and the ratio of competing RNA to labeled RNA must be high enough that the labeled RNA represents a negligible fraction of the total. Competing RNA is not necessarily synonymous with unlabeled RNA, since a large part of the unlabeled RNA may consist of sequences unrelated to the labeled RNA and not participate in the competition under study.

There are 3 ways to alter the experimental design to favor reaching an end point: (a) add more competing RNA (without exceeding the solubility limit at the incubation temperature); (b) reduce the amount of DNA or labeled RNA or both, depending on which criterion may not have been met (this reduces the sensitivity of the experiment unless labeled RNA of higher specific activity is used); or (c) use nRNA instead of total RNA since nRNA contains all base sequences present in total RNA but is enriched for nonribosomal transcripts and so contains a higher concentration of competing sequences.

A combination of Methods b and c was selected to complete the comparison of liver with tumor RNA (Chart 2). The competition curve with liver nRNA drops to an insignificant level, indicating that liver RNA is able to compete for all of the DNA sites complementary to primary hepatoma RNA. This means that the liver is transcribing RNA's from all of the gene families which are active in the tumor, and therefore no additional gene families were derepressed during malignant transformation.

Comparison of Primary and Transplantable Tumors. Bile ducts constitute a far smaller fraction of the total cell population in the normal liver than in the induced tumors that we have studied. This could be the cause of the slope difference noted in Chart 1, if the 2 cell types contain different proportions of various RNA sequences. As a test of this, liver RNA was enriched for ductal components by the addition of 0.5 and 1.0 mg of RNA from Novikoff hepatoma, which has been shown to lack microbodies and to contain cytoplasmic filaments characteristic of bile duct cells (10). This mixture is a much more efficient competitor than either liver or Novikoff tumor alone, as indicated by the steeper slope of the curve (Chart 3), supporting the hypothesis that the quantitative difference between liver and primary tumor RNA's is related to a difference in distribution of cell types.

A direct corollary of this hypothesis is that a tumor which is more enriched for parenchymal cells should not compete as efficiently as the primary tumor. Morris hepatoma 5123 appears both histologically and ultrastructurally to be parenchymal. The ability of its RNA to compete against the primary tumor RNA (Chart 3) reflects this, and the competition is incomplete. This suggests that Tumor 5123 might be closer to the normal liver in its distribution of cell types than are the primary tumors.

Comparison of Liver with Transplantable Tumor. The comparison of normal liver with tumor was repeated with labeled RNA of Morris hepatoma 5123 (Chart 4). Again liver nRNA competed completely, indicating that no gene families are activated in this tumor either and supporting the idea that genetic derepression is not a significant factor in the mechanism of carcinogenesis.

The difference between the slopes of the curves with total RNA was far less than in Chart 1, confirming that the distribution of RNA's in this tumor is more similar to that of normal liver than are the primary tumors.

Comparison of Cytoplasmic RNA's. Charts 2 and 4 include the curves of competition by tumor and normal cytoplasmic RNA's.
Search for Gene Derepression in Hepatomas

RNA's. A significant difference is demonstrated. Tumor cytoplasmic RNA competes efficiently with all components of labeled tumor RNA. Both primary and transplantable tumors lack RNA species restricted to the nucleus. Although the curves with normal liver cytoplasm do not show clear plateaus in these experiments, the existence of nucleus-restricted RNA in normal mouse liver has been proven (6), and similar experiments do plateau when extended (19). This difference in transport of RNA out of the cell nucleus cannot be attributed entirely to rapid growth rate, since even liver regenerating after 70% hepatectomy has nucleus-restricted RNA (6).

DISCUSSION

Competition hybridization can be used to show differences between populations of RNA molecules. With mammalian DNA, competition experiments cannot be used to identify like RNA species because of the large fraction of related genes whose base sequences are similar enough to cross-react (2). Also, the fraction of the mammalian genome which consists of single-copy DNA sequences cannot be studied by competition hybridization. This is the result of operational difficulties, e.g., the concentration of RNA and DNA required for a reasonable reaction rate are near saturation and preclude the addition of competing RNA. The results of our competition studies must be viewed with these factors in mind.

These studies show only that additional gene families are not activated in the tumors studied and do not preclude derepression of individual genes within a family which already has active members. This technique has been used to demonstrate changes in the spectrum of active gene families during development of fetal mouse liver (5), following partial hepatectomy in adult mice (4) and in early sea urchin (21) and toad (7) embryonic development. The absence of these gross differences between the RNA's of induced tumors and host liver indicates that in this sense malignant transformation appears not to represent a reversion to an embryonic state.

Since the labeled RNA in these studies was derived from tissue culture passage of an induced tumor, it can be argued that we may have lost RNA coded by genes not expressed in culture conditions. It is indeed possible that tissue culture of tumors may represent a process modifying gene expression, but cancer-related gene expression seems not to be lost since a number of the induced tumors, including the Morris hepatomas, can be passaged back and forth from tissue culture to animal host.

These experiments confirm and extend the observations of others (8), that host liver contains all the RNA species present in transplantable Morris hepatomas. Our data have shown that primary hepatomas induced by an azo dye also lack abnormally derepressed gene families. The alteration in primary hepatomas of the regulatory mechanism concerned with selective transport of RNA to the cell cytoplasm (6, 12, 16, 17) may be an important step in tumorigenesis. The characteristics of tumors which have been explained by the derepression theory can just as well be caused by translation of gene messages which are normally restricted to the nucleus and therefore sequestered from the sites of protein synthesis. These include tumor antigens, enzymes appropriate to an earlier stage of development, and the components needed for cell division. The RNA's which are restricted to the nucleus in normal liver but released to the cytoplasm in hepatomas are being characterized.

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R. W. Shearer and E. A. Smuckler


Figs. 1 to 3. Electron micrographs of sections stained with uranyl acetate and lead. X 15,800.

Fig. 1. Tumor DS-2A. Parenchymal cell surrounded by undifferentiated cells.

Fig. 2. Tumor DS-10A. Undifferentiated cell with fat droplets and abnormal mitochondria.

Fig. 3. Tumor DS-1A. Ductal cells with microfibrils and convoluted cell membranes.
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