Recognition of Altered Patterns of Messenger RNA Synthesis in a Mouse Hepatoma*

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SUMMARY
Patterns of messenger RNA synthesis in a transplanted spontaneous BR6 mouse hepatoma have been compared with those of normal BR6 mouse liver by countercurrent distribution. The liver patterns showed little variation, whereas in the hepatoma there was progressive alteration in the messenger RNA profile with progression of the tumor. Evidence from countercurrent distribution profiles of liver and hepatoma DNA and from comparative rates of messenger RNA synthesis suggests that less of the tumor genome is being transcribed into messenger RNA. From the messenger RNA profiles on countercurrent distribution it is evident that, of those regions of the hepatoma genome which are undergoing transcription, many are different from those being transcribed in the normal liver.

Genetic changes involved in the transition from normal to neoplastic cells have been inferred generally from changes in growth pattern, in some cases from observed chromosome aberrations, and in many instances from alterations in enzyme patterns. Quantitative variations in enzyme levels, the absence of an enzyme in a given tumor, and the failure to induce it do not distinguish between structural and regulatory gene mutations, nor indeed do they necessarily indicate the occurrence of true mutation. Such changes may also be explained in terms of altered transcription of given DNA base sequences (15). Qualitative changes in enzyme structure or kinetics in tumor cells frequently appear to indicate underlying mutation in the structural gene concerned, but the change may represent an induced isozyme produced minimally in the original normal cell or an alteration in translation of messenger RNA into specific protein, analogous to the effects of suppressor mutations in bacteria (4).

What is required ideally is a means of direct “reading” of the genome of the tumor cell and the cell of origin, at the levels of the total genome, the effective genome, and the facultative genome (5). This is especially true with respect to regulatory loci, the action of which is far from clear in higher organisms, in contrast to the accumulation of data supporting the postulates of Jacob and Monod (9) for microorganisms.

At present this is far from possible at the desirable level of DNA base sequences. However, analysis of patterns of messenger RNA (mRNA) synthesis does permit assessment in less precise terms of the expression of the genome under a given set of conditions. This has been approached in a variety of ways: in bacteria by chromatography on methylated albumin kieselguhr columns (14, 16, 17) and by base sequence complementarity through hybrid formation with homologous or heterologous DNA in DNA-containing columns (1, 2, 6); in mammalian tissues by hybrid formation (8) and by countercurrent distribution (CCD). This latter method allows direct recognition of altered patterns of messenger RNA synthesis and has provided a means of identifying tissuespecific mRNA patterns.

In the present studies CCD has been used to identify altered patterns of mRNA synthesis in a transplanted, spontaneous mouse hepatoma (BR6). This tumor, retaining certain normal metabolic features such as synthesis of glycogen, in some respects conforms to the definition of a "minimal deviation hepatoma." As such it is a useful model for analysis of patterns of messenger RNA synthesis in cells of established tumors.

MATERIALS AND METHODS

Materials.—The BR6 hepatoma is a spontaneous parenchymal cell tumor of mouse liver which maintains at least some aspects of normal liver metabolic function, notably glycogen synthesis. In transplanted form it is

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slow-growing compared with transplanted azo dye-induced hepatomas and is of relatively low-grade malignancy. From about 6 weeks onward there is increasing central necrosis not infrequently accompanied by hemorrhage into the tumor. In the present investigations the hepatoma was studied under conditions of serial transplantation into male or female Br6 mice 6-8 weeks old, the tumor having been maintained by transplantation for 5 years at the time of these experiments. Tumors were used 4-10 weeks after transplantation. Animals were killed by breaking their necks, and liver and hepatoma were removed immediately from each animal and frozen on dry ice or in liquid nitrogen. The respective tissues were pooled from at least four mice in each experiment.

Histology.—Histological sections showing the presence of glycogen in the hepatoma cells were prepared on tumors fixed in Bouin’s fluid. Staining was with Best’s carmine, counterstaining with Ehrlich’s hematoxylin. Control sections were stained after treatment with saliva (Figs. 1, 2).

Pulse-labeling.—In experiments on mRNA pulse-labeling was carried out by intraperitoneal injection of 100-250 μc. of uridine-H³ per mouse. Pulse time was assessed from time of injection to freezing of the tissues. The usual pulse time was 20 minutes, when maximal incorporation of label into the rapidly labeled RNA fraction has been found to occur (11).

Extraction of mRNA and DNA.—Extraction of the rapidly labeled RNA containing most of the mRNA with a high turnover rate was carried out essentially as described for rat liver (11). Ribosomal and soluble RNA were first removed by extraction with 0.5 per cent naphthalene-1,5-disulfonate/90 per cent phenol/0.1 per cent 8-hydroxyquinoline and discarded in the aqueous phase. DNA and mRNA were then extracted from the insoluble phenolic interface with 6 per cent 4-aminosalicylate/90 per cent phenol/0.1 per cent 8-hydroxyquinoline, precipitated from the aqueous phase with ethanol, washed, and dried. This interfacial DNA/RNA was then used to study mRNA patterns.

DNA was extracted and purified by the 4-aminosalicylate method (12).

Sucrose density gradient centrifugation.—Analysis of mRNA preparations (1 mg. DNA/RNA) was carried out by centrifugation in sucrose gradients of 5-20 per cent sucrose in 0.01 m sodium acetate, pH 5, at 24,000 r.p.m. at 4° C. for 14 hours in a Spinco SW 25 rotor. Fractions of 10 drops were collected from the bottom of the tube, diluted with water to 1.5 ml., and the nucleic acids were extracted and counted by liquid scintillation spectrometry as described previously (11).

Countercurrent distribution of mRNA.—Solvent systems for CCD were similar to those developed for ribosomal RNA (13), except that trilithium citrate was substituted for tripotassium citrate in the aqueous phase. The proportions in the CCD system employed here for mRNA were: organic mixture, 28 volumes; amine solution, 12 volumes; 0.033 m trilithium citrate, 13.5 volumes; and water, 38.5 volumes (solvent system 150/13.5).

Interfacial DNA/RNA (1.5 mg.) was distributed in this system over 100 transfers. The DNA/RNA was first dissolved in 3 ml. of each phase of a 150/2 system, then transferred to the lower phase of the final solvent system via a two-tube gradient of increasing lithium ion concentration. After 100 transfers 1 ml. of ethanol was added to each tube to give one phase, and radioactivity was measured in 2-ml. aliquots by liquid scintillation spectrometry (10 ml. of scintillation mixture).

Countercurrent distribution of DNA.—Native DNA from liver or hepatoma was distributed in the same solvent system as for mRNA, but containing 11.7 volumes of trilithium citrate and 40.3 volumes of water (solvent system, 150/11.7). One mg. was distributed over 80 transfers (2 ml. each phase), ethanol added as above, and the absorption read at 260 μm (10).

Chromosome counts on Br6 hepatoma.—Animals were given injections intraperitoneally of 0.04 per cent (w/v) colcemid (CIBA), a portion of the tumor was removed, the cells were freed from stroma, treated for 20 minutes with 1 per cent citrate solution, then fixed for 1 hour in 45 per cent acetic acid. After acid hydrolysis (6 min. in NHCl at 60° C.), the tissue was stained with Feulgen, and chromosome counts were carried out on squash preparations.

RESULTS

Rate of mRNA synthesis.—The relative rates of incorporation of uridine-H³ into rapidly labeled mRNA in a 20-minute pulse in mouse liver and hepatoma in a series of experiments are listed in Table 1. These are expressed as counts/min/A₄₀₀₀ m, the absorption representing mainly DNA. The rate of synthesis of mRNA as judged by uridine incorporation was consistently higher in liver than in the hepatoma, at all time periods of tumor growth studied.

Sucrose gradients of mRNA.—Chart 1 shows the patterns obtained by centrifugation of mRNA samples in sucrose density gradients. The tumor patterns were less consistent than those for liver but mRNA’s from both tissues had a wide range of s values which, for mouse liver (12-40 s) were essentially the same as for rat liver (11). The hepatoma mRNA had profiles different from those for liver but with a similar range of s values. These findings indicate that little or no degradation of mRNA occurred.
during preparation and are consistent with considerable heterogeneity in size of mRNA's in both tissues.

**Countercurrent distribution of mRNA.**—Mouse liver mRNA's showed relatively consistent distribution patterns on CCD in a series of experiments (Chart 2). DNA, rRNA, and sRNA remain largely in the first twenty tubes in the solvent system used. The radioactive patterns obtained for liver mRNA were seen only under the standardized conditions of diet and methods of preparation that have been used. The CCD patterns of tumor mRNA taken at 4, 6, and 8 weeks showed considerable heterogeneity in profile, little consistency among themselves, and great differences from the patterns of liver mRNA. There was a trend away from the liver pattern the longer the time of growth after transplantation, but the distribution at no time closely resembled that of liver in all respects.

**Countercurrent distribution of DNA.**—Native DNA from mouse liver and hepatoma (6 weeks) gave CCD patterns illustrated in Chart 3, in solvent system 150/11.7. That for mouse liver, with a narrow initial peak and a large number of subsequent peaks, follows the same general pattern observed in other mammalian tissues (10). The initial peak represents DNA present mainly as firmly hydrogen-bonded double helices, whereas the remainder represent DNA with partly separated strands, denaturation being greatest in those molecules traveling furthest in the organic phase. In the hepatoma the same initial peak was observed, but there was less spreading of the remainder, indicating the presence of fewer molecules.

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CHART 1.—Sedimentation profiles obtained by centrifugation in sucrose gradients of rapidly labeled mRNA from BR6 livers and hepatomas, from the same animals at varying times after transplantation of the tumors.

CHART 2.—Profiles obtained by countercurrent distribution of rapidly labeled mRNA from BR6 livers and hepatomas from the same animals at varying times after transplantation of the tumors. Solvent system: 150/13.5, 100 transfers.

CHART 3.—Profiles obtained by countercurrent distribution of DNA from BR6 livers and hepatomas from the same animals, 6 weeks after transplantation of the tumors. Solvent system: 150/11.7, 80 transfers.

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with a high degree of strand separation. The degree of spreading of the partly denatured DNA from the hepatoma varied from preparation to preparation but appeared always to be less than for liver DNA.

**Chromosome counts on BR6 hepatoma.**—Chart 4 shows a histogram of chromosome numbers on a 6-week-old hepatoma. The majority of cells had 40 or 80 chromosomes, but considerable heteroploidy was evident, the range being from 7 to 96 per cell.

**DISCUSSION**

Evidence for markedly differing patterns of messenger RNA synthesis in a BR6 mouse hepatoma compared with mouse liver has been derived from sucrose gradient centrifugation and from countercurrent distribution. The mRNA in these DNA/RNA preparations consisted mostly of free mRNA with small amounts present as ribonuclease-resistant RNA/DNA hybrid or associated with DNA-protein complex: the comparisons are as much between patterns of mRNA synthesis as between mRNA fractions from liver and hepatoma.

In our hands reproducibility of minor peaks of rapidly labeled RNA on sucrose gradients has not been sufficiently good to warrant precise interpretation of small differences. The sedimentation characteristics of hepatoma and liver mRNA in the present studies, however, did reveal differences in general profile, although there was similarity in the range of s values. This suggests that both mouse liver and hepatoma mRNA's are heterogeneous in size (7, 11).

Fractionation by CCD, in which nucleotide base composition plays a major role, has been found to give a high degree of reproducibility for a given preparation of mRNA and for different preparations made from a given tissue type under standardized conditions. CCD has revealed striking differences between the patterns of mRNA synthesis in liver and hepatoma. In contrast to the very similar liver patterns, the CCD profile for the hepatoma mRNA varied with different stages of tumor growth. The indication from CCD profiles is that in the hepatoma there is both diminished synthesis or absence of certain groups of mRNA's produced by liver, and synthesis of mRNA's absent from or produced to a much less extent by liver. Any effects due to variation in quantity of protein bound would be most noticeable in the first ten to twenty tubes, since DNA-protein and RNA-protein complexes tend to remain in the aqueous phase. Detailed chemical and biological characterization is required to delineate the extent of identity and difference between the mRNA fractions from liver and hepatoma.

The presence in the BR6 hepatoma of considerable heteroploidy makes interpretation of the pattern of messenger RNA synthesis in the tumor somewhat difficult. The altered patterns of mRNA may have resulted from variations in the activities of the different chromosomes in the cells, and the progressive change in the mRNA pattern may be an indication of the variation of mRNA synthesis with respect to time. These questions cannot be clarified at the present time. It is relevant, however, to note that, although the over-all rate of mRNA synthesis in the tumor is apparently greatly diminished compared with that in the liver, there appears to be considerable emphasis on the synthesis of groups of mRNA's which are present in very small amounts or are absent in the normal liver. This is strongly suggestive of induction of mRNA's not normally produced in liver; a situation which is unlikely to derive from heteroploidy alone.

CCD behavior of the hepatoma DNA presents features which are helpful in understanding the functioning of the tumor genome. The initial peak (about tube 10) represents DNA in which the strands are almost completely linked by hydrogen bonds. The DNA molecules present in tubes 20-80 are characterized by an increasing degree of strand separation which is related to the distance traveled in the organic phase, the strand separation being particularly marked in tubes 60-80. There is clearly much more of this latter material in the normal liver than in the tumor. In the present solvent system natural DNA/RNA hybrids, judged by that portion of the mRNA which is resistant to ribonuclease, were associated with those molecules of DNA which exhibit a degree of strand separation and not with the initial DNA peak in which strand separation is minimal. Thus, the DNA pattern on CCD, together with the observed decrease in rate of mRNA synthesis, is consistent with the transcription of a reduced amount of the genome in the hepatomas compared with that in the liver.

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**CHART 4.**—Histogram of chromosome numbers in squash preparations of a BR6 hepatoma 6 weeks after transplantation.
tion alone may not be an adequate criterion for mRNA synthesis, since this may be affected by the rate of uptake, utilization, and pool size of RNA precursors. Polyploidy could also influence the rate of synthesis of mRNA per unit DNA. As already discussed, the appearance of "new" groups of mRNA’s suggests that some of those regions of the tumor genome which are being used for transcription are different from those of the liver. Further studies on the regulation of synthesis of individual groups of mRNA’s thus fractionated and their chemical and biological characterization may be expected to reveal some of the mechanisms involved in the progressive alteration of expression of the liver cell genome in the transition to hepatoma cell.

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