# Alterations in Polyadenylated Messenger Ribonucleic Acid from Free and Total Polysomes of a Rat Hepatoma†

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ABSTRACT: We examined the homology between polysomal polyadenylated ribonucleic acid (mRNA) populations of hepatoma 252, a tumor which is deficient in the synthesis of plasma proteins, and those of normal and regenerating rat liver. Hybridization of polyadenylated mRNA populations with homologous or heterologous complementary deoxyribonucleic acids showed that mRNA from total and free polysomes from hepatoma 252 lack sequences which are present in normal or regenerating liver. Although there are obvious differences in the abundance of sequences between tumor and normal or regenerating liver polysomal mRNA, we did not detect, with the techniques used in this work, tumor-specific sequences. Analysis of hybridization curves using derivative plots did not reveal the presence in tumor mRNA of a high complexity class not present in normal liver. We conclude that alterations in mRNA populations of free and total polysomes of this tumor primarily reflect processes of genetic restriction rather than the derepression of previously unexpressed genes.

Cells of mammalian livers, which in the adult animal rarely replicate, readily respond to losses of the organ mass, triggered experimentally by partial hepatectomy, by undergoing hypertrophy and hyperplasia (Bucher & Malt, 1971). In addition to this compensatory growth response, it is also relatively easy to induce neoplastic growth in the liver of adult rats. During the process of hepatocarcinogenesis produced experimentally by many chemical agents, there are drastic changes in the cellular composition of the liver, and a number of phenotypic changes take place. In contrast to hepatocarcinogenesis, the process of compensatory growth does not cause major histological alterations in the organ, and despite the intense proliferative response, growth ceases when the original organ mass is restored.

To properly understand the mechanisms which initiate and regulate compensatory and neoplastic growth processes, it is important to analyze the patterns of gene expression in both of these conditions. The liver is an excellent system for these studies because one can examine and compare some characteristics of genomic expression in the normal organ in which cells are metabolically active but have a negligible rate of cell proliferation with those (a) in regenerative growth, during which hypertrophy and hyperplasia of normal hepatocytes take place, (b) during induction of primary hepatocellular carcinomas which includes a range of preneoplastic and neoplastic changes, and (c) in transplantable hepatocellular carcinomas (hepatomas), which are malignant liver tumors that can be transplanted from animal to animal by inoculation of the tumor cells into extrahepatic sites.

Earlier work reported in the literature suggested that during liver regeneration there are marked increases in the fraction of the genomic DNA[1] which is transcribed into RNA (Church & McCarthy, 1967). However, work done in one of our laboratories (Tedeschi et al., 1978), as well as that of others (Wilkes et al., 1979; Grady et al., 1979), indicates that the percentage of single-copy DNA expressed during liver regeneration does not differ from that of normal liver. Moreover, the sequence complexities of polyadenylated polysomal mRNA of normal and regenerating liver are similar (Colbert et al., 1977), and there is extensive, if not complete, homology between these mRNA populations (Wilkes et al., 1979; Scholla et al., 1980). Thus, although there is a large increase in the amount of polysomal poly(A)$^+$ mRNA in regenerating liver (Atryzek & Fausto, 1979), qualitative changes in polysomal mRNA have not been demonstrated in the most recent work (Wilkes et al., 1979; Scholla et al., 1980).

Atryzek et al. (1980) in one of our laboratories investigated the characteristics of polysomal poly(A)$^+$ mRNA populations in livers of rats kept on a carcinogenic diet which contains ethionine and is choline deficient. These authors did not detect tumor-specific sequences in polysomal polyadenylated mRNA extracted from livers with extensive cellular alterations or from those containing carcinomatous nodules. Knöchel et al. (1980) reported similar observations in rats fed the carcinogen 3′-methyl-4-(dimethylamino)azobenzene for 17 weeks. Polysomal poly(A)$^+$ mRNA from the liver of these animals had the same sequence complexity and appeared to be completely homologous to polysomal mRNA of normal livers. It is possible, then, that both regenerative growth of the liver and the cellular changes which lead to the development of primary hepatic tumors may take place without major qualitative changes in gene expression. Although one cannot entirely exclude, at this time, the existence of some qualitative changes in mRNA populations, alterations in the abundance of various RNA sequences may be sufficient to initiate and maintain these growth processes.

A complete picture of gene expression in neoplastic liver requires an examination of the homology between mRNA populations of transplantable hepatomas with those of normal and regenerating livers. Given the morphological and metabolic characteristics of transplantable hepatomas, one might expect to find changes in gene expression manifested by the existence of tumor-specific RNA sequences or by the absence of mRNAs present in normal liver. A major proportion of

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[1] Abbreviations used: DNA, deoxyribonucleic acid; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); poly(A)$^+$ mRNA, polyadenylated messenger ribonucleic acid; NaDodSO$_4$, sodium lauryl sulfate; $R_0t$, product of the initial concentration of nucleotides in moles per liter multiplied by the incubation time in seconds; cDNA, complementary DNA; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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liver mRNA codes for the synthesis of serum proteins. The intracellular levels of these mRNAs may change drastically in neoplasms, probably as a consequence, rather than a cause, of the neoplastic process. Consequently, it might be easier to find tumor-specific sequences directly related to neoplastic transformation, if they exist, in a system which lacks at least some of the mRNAs coding for exportable proteins. For this reason, we attempted to detect tumor-specific sequences or the loss of mRNA sequences in transplantable hepatomas by examining the homology between polysomal poly(A)$^+$ mRNA of hepatoma 252 and polysomal poly(A)$^+$ mRNA of normal and regenerating liver. This tumor is deficient in the synthesis of serum proteins (Becker et al., 1972) and does not appear to contain mRNAs for exportable proteins such as albumin, $\alpha$-fetoprotein, and $\alpha$-2-$\mu$-globulin (Sell et al., 1979; Feigelson & Kurtz, 1978). Since free polysomes are involved in the synthesis of internal cell proteins, we isolated mRNA populations from both total and free polysomes of the hepatoma 252 and compared them with polysomal mRNA populations from normal adult livers (sham-operated rats) and from the regenerating livers of adult or very young rats ("weanlings").

## Experimental Procedures

*Animals and Surgical Procedures.* Animals with hepatoma 252 were from the colony maintained at the M.D. Anderson Hospital and Tumor Institute (F.F.B.). Rats were inoculated intramuscularly with the tumors in bilateral sites. The animals were shipped to Brown University (N.F.) where they were kept in a temperature-controlled room with alternating light–dark cycles. Killing was done ~10 days after tumor inoculation, when tumor size was around 2 cm in diameter. Necrotic portions of the tumor were discarded. Partial hepatectomies were performed on weanling rats (50–70 g) or in animals weighing 140–180 g (Charles River Laboratories, Holtzman strain male rats) by the method of Higgins & Anderson (1931). Weanling and adult rats were killed 22 and 24 h after the operation, respectively, which correspond to the times of maximal DNA synthesis for animals of these ages (Bucher et al., 1964). RNA preparations referred to as "normal liver" RNA were obtained from sham-operated rats, that is, animals which were laparotomized and their livers manipulated but not removed. Surgical procedures were done under continuous oxygen–ether anesthesia as previously described (Fausto & Butcher, 1976). Food was withdrawn 14–16 h prior to killing which was done between 9 and 11 a.m.

*Isolation of Total Polysomes.* Total polysomes from normal and regenerating liver were isolated exactly as previously described (Scholla et al., 1980). For the isolation of polysomes from hepatoma, the tumors were excised immediately after decapitation and rapidly washed, cleaned, and minced in cold solution H [25 mM Tris (pH 7.4), 250 mM KCl, 40 mM NaCl, 7.5 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 250 $\mu$g/mL heparin, and 0.25 M sucrose]. The tissue was homogenized with a motor-driven Teflon homogenizer in 5 volumes of solution H at 200–500 rpm. The homogenate was adjusted to 0.5% each of Triton X-100 and deoxycholate by dropwise addition, with stirring, of a freshly made solution of 10% Triton X-100 and 10% deoxycholate in 25 mM Tris-HCl, pH 7.5. After being stirred for 2 min, the homogenate was centrifuged at 27000$g$ for 5 min at 2 °C. Heparin was added to the postmitochondrial supernatant to a final concentration of 1 mg/mL, and then 0.06 volume of the Triton X-100–deoxycholate solution was added dropwise, with stirring, to a final concentration of 0.6%. This extract was layered over discontinuous sucrose gradients of 0.5, 1, and 2.5 M sucrose in solution G [25 mM Tris (pH 7.5), 25 mM NaCl, 5 mM $MgCl_2$, and 250 $\mu$g/mL heparin] and centrifuged at 25000 rpm for 4 h at 4 °C in a Beckman SW 27 rotor. Polysomes were collected from the 2.5–1 M sucrose interface by puncturing the tube with a needle and syringe. Polysomes from each tube were pooled, fast frozen in ethanol–dry ice, and stored at −70 °C.

*Isolation of Free Polysomes.* Livers or tumors were washed, cleansed, and minced in cold modified A solution [20 mM Tris (pH 7.6), 10 mM magnesium acetate, 40 mM NaCl, 25 mM KCl, 250 $\mu$g/mL heparin, 6 mM 2-mercaptoethanol, and 0.125 M sucrose]. The tissue was homogenized in 4 volumes of modified solution A at 1500 rpm and then centrifuged at 100000$g$ for 12 min at 4 °C. Heparin was added to the postmitochondrial supernatant to a final concentration of 1 mg/mL. The extract was layered onto discontinuous sucrose gradients (1 and 2 M sucrose in solution G) and centrifuged for 19–20 h at 105000$g$ at 4 °C to pellet the polysomes. The pellets were rinsed with TNM buffer [50 mM Tris (pH 7.1), 25 mM NaCl, and 5 mM $MgCl_2$], drained, and stored at −70 °C.

*Extraction of Polysomal Polyadenylated mRNA.* Extraction of RNA from all preparations, with the exception of tumor total polysomes, and isolation of polyadenylated RNA on poly(U)–Sepharose columns were exactly as previously described (Scholla et al., 1980). The extraction procedure was slightly modified for tumor total polysomes because they were not pelleted. $NaDodSO_4$ was added to the sucrose solution containing the suspended polysomes to a final concentration of 2%. The solution was then brought to 0.1 M NaCl and extracted 2 times with TNM saturated phenol–chloroform (1:1 v/v) and 2 times with chloroform–isoamyl alcohol (24:1 v/v). NaCl was added to the final aqueous phase to a concentration of 0.2 M, and the nucleic acids were precipitated in 2 volumes of 95% ethanol. The precipitate was dissolved in NETS buffer [100 mM NaCl, 10 mM Tris (pH 7.5), 10 mM EDTA, and 0.2% $NaDodSO_4$], loaded onto a poly(U)–Sepharose column, and eluted with 90% formamide in NETS. After passage through poly(U)–Sepharose, all RNAs were precipitated with ethanol, dissolved in 10 mM NaCl, and passed through a Chelex 100 (Bio-Rad Laboratories) column. After checking the absorbance of the recovered RNA at 230, 260, and 280 nm, the RNA was precipitated with ethanol 2 times, dissolved in $H_2O$, and stored at −70 °C.

*Preparation of [$^3H$]cDNA.* [$^3$H]cDNAs complementary to polyadenylated RNAs from total and free polysomes from tumor tissue and from normal and regenerating liver were synthesized exactly as previously described (Scholla et al., 1980) by using avian myeloblastosis virus reverse transcriptase (gift of Dr. J. W. Beard, Life Sciences Inc.). The labeled cDNA was passed through a column of Sephadex G-50 over Chelex 100. The excluded fractions were pooled, treated with 0.5 N NaOH for 5 min at 100 °C, and neutralized. Carrier tRNA was added to 50 $\mu$g/mL, the cDNA was precipitated with ethanol, and the precipitate was dissolved in $H_2O$ and stored at −70 °C after fast freezing in ethanol–dry ice.

*Messenger RNA–cDNA Hybridization.* Appropriate amounts of polyadenylated RNA were mixed with [$^3$H]cDNA at RNA/DNA ratios of $5 \times 10^2$–$4 \times 10^4$. The nucleic acids were mixed, lyophilized to dryness, and dissolved in hybridization solution [0.24 M sodium phosphate (pH 6.9), 1 mM EDTA, and 0.05% $NaDodSO_4$]. The hybridization mixtures were incubated in sealed, sterile, siliconized capilary tubes at 70 °C after boiling for 5 min to fully denature the nucleic acids. Incubation times were no longer than 94 h, and RNA concentrations used to reach the appropriate $R_0t$ values were

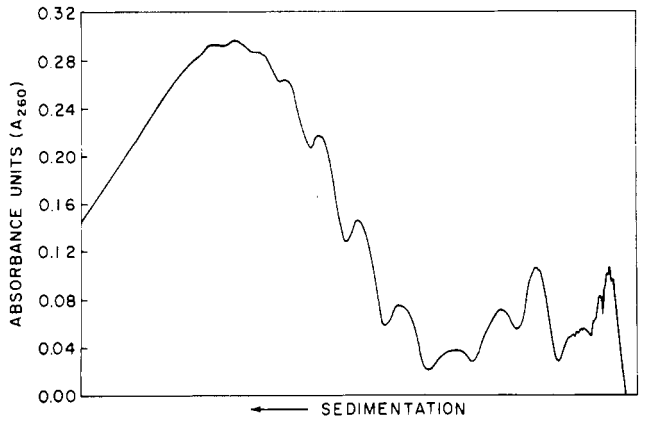


FIGURE 1: Polysomal profile of total tumor polysomes. Polysomes were prepared as described under Experimental Procedures and centrifuged on a 15–30% sucrose gradient for 3 h at 25 000 rpm in an SB-110 rotor of a B-60 International ultracentrifuge. The direction of sedimentation is from right to left as indicated by the arrow. The absorbance at 260 nm, which was continuously recorded with a flow cell, is shown on the ordinate.

50, 1000, or 4000 μg/mL. All reactions were terminated by immersing the capillary in ethanol–dry ice. For determination of the percentage of cDNA hybridized, the contents of each capillary was expelled into a tube containing $S_1$ solution (50 mM sodium acetate, pH 4.5, 1 mM $ZnSO_4$, 100 mM NaCl, and 10 μg/mL heat-denatured calf thymus DNA). From each tube two samples were assayed; one was digested with 1000 units of nuclease $S_1$ from *Aspergillus oryzae* (Boehringer Mannheim) at 37 °C for 1 h and the other incubated without $S_1$. The samples were chilled, made 200 μg/mL with carrier DNA, precipitated with trichloroacetic acid, and collected on Whatman GF/C filters. Radioactivity was determined for each sample, and the percentage of [$^3$H]cDNA hybridized was calculated as (cpm in $S_1$ treated sample)/(cpm in untreated sample) × 100. Zero time values for the hybridization (1–3%) were used as background and have been subtracted from all data presented.

## Results

*Heterologous Hybridization of cDNA with Polyadenylated mRNA from Total Polysomes.* Tumor polysomes were isolated without pelleting the material. The profiles obtained after centrifugation in sucrose gradients were similar to those of polysomes isolated from normal or regenerating liver and show that the polysomes are not degraded (Figure 1).

The average length of the mRNA obtained from normal, regenerating liver and tumor mRNA was approximately 1500–1800 nucleotides. These values are similar to those reported previously by Atryzek & Fausto (1979), Reiners & Busch (1980), and Scholla et al. (1980). The size of the cDNAs transcribed was determined as previously described (Colbert et al., 1977; Scholla et al., 1980). Complementary DNA transcribed from polyadenylated mRNA of normal and regenerating liver and from tumor had similar size distributions corresponding to a modal length of 600–800 nucleotides.

Complementary DNA synthesized from normal liver polysomal poly(A)$^+$ mRNA was hybridized with polyadenylated mRNA extracted from tumor total polysomes. This curve is shown in Figure 2a in conjunction with the homologous hybridization of normal liver cDNA with its own template mRNA. Two features of the curves presented in Figure 2a should be noted: (a) the heterologous reaction (solid line) lags behind the homologous reaction at the very low $R_0t$ values, which correspond to the hybridization of very abundant mRNAs; (b) the saturation level of the homologous reaction is ~10% higher than that of the heterologous curve. These curves suggest that the amount of polysomal mRNA sequences present in normal liver, especially those from very abundant mRNAs, decreases in tumor RNA and that the tumor lacks some of these sequences.

To determine if tumor polysomal mRNA contains sequences which are not present in normal liver, we hybridized cDNA transcribed from tumor polysomal polyadenylated mRNA with mRNA obtained from normal liver polysomes (Figure 2b, open circles). This curve has the same overall shape, but the hybridization is slower than that for the homologous reaction (tumor cDNA hybridized with tumor mRNA, solid line in Figure 2b). Since there is considerable overlap between the hybridization values at the plateau level of these two curves, it is not possible to conclude that there are hepatoma-specific polysomal mRNA sequences which are not present in normal liver polysomes. However, it is obvious that some of the tumor mRNA sequences are less abundant in the normal liver.

To further examine the question of the existence of tumor-specific sequences in polysomal poly(A)$^+$ mRNA of hepatoma 252, we compared tumor mRNA sequences with those of polysomal mRNA of regenerating liver of weanling rats. Regenerating liver mRNA was hybridized with tumor cDNA, and the corresponding curve is shown by the dashed line with

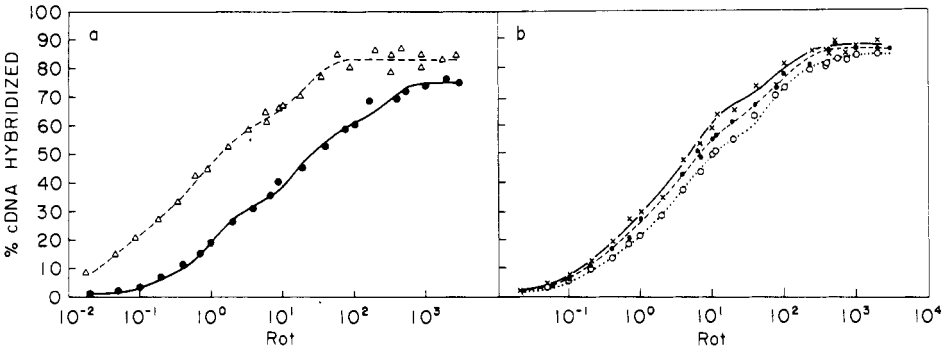


FIGURE 2: Hybridization of normal and tumor [$^3$H]cDNA to homologous and heterologous mRNAs. See Experimental Procedures for hybridization conditions. All hybridization curves were drawn by using a computer program developed by Pearson et al. (1977). The notation used in this and other figures is as follows: "normal cDNA" = cDNA transcribed from poly(A)$^+$ polysomal mRNA from livers of 24-h sham-operated rats; "tumor cDNA" = cDNA transcribed from poly(A)$^+$ polysomal mRNA from hepatoma 252; "weanling RNA" = poly(A)$^+$ polysomal mRNA isolated from polysomes of partially hepatectomized weanling rats. The mRNA concentrations were 50 μg/mL and 1 and 4 mg/mL, and the RNA/cDNA ratios were 5 × $10^2$–$10^4$. The extent of hybridization was determined by digestion of the reaction mixture by $S_1$ nuclease. Zero time values of 1–3% have been subtracted: (a) hybridization of normal [$^3$H]cDNA to its template mRNA (Δ) and to mRNA isolated from total tumor polysomes (●); (b) hybridization of tumor [$^3$H]cDNA to its template mRNA (×), to weanling mRNA (●), and to normal mRNA (○).

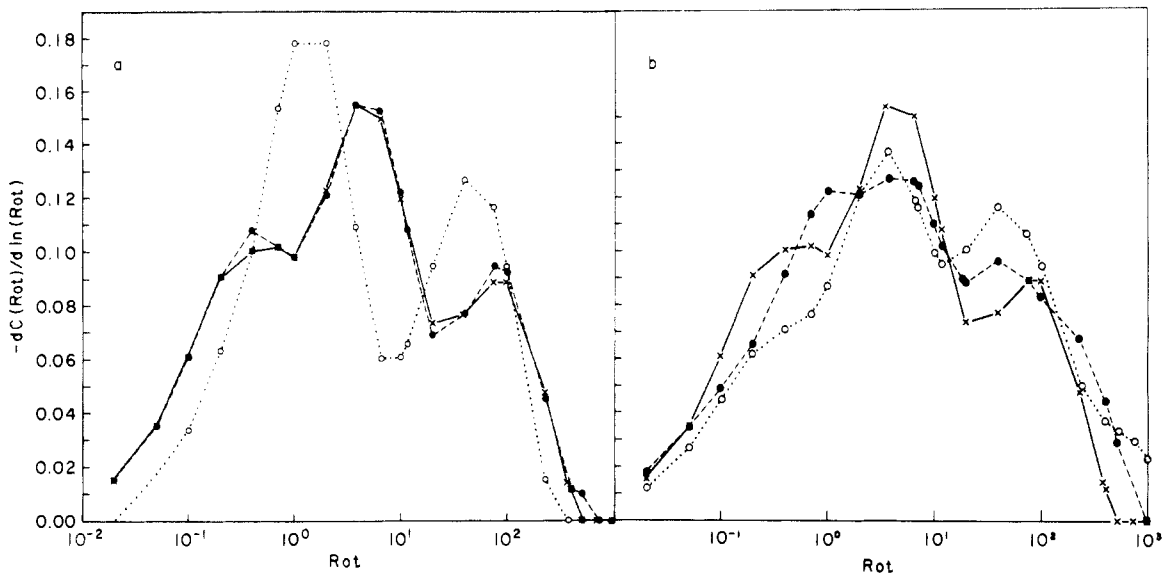


FIGURE 3: Derivative plot of hybridization of total polysomal tumor [$^3$H]cDNA to homologous or heterologous mRNAs. (a) Derivatives for the homologous hybridization curve of total polysomal tumor cDNA with its template mRNA (shown by the solid line in Figure 2b) were calculated by the method described by Quinlan et al. (1978). The curves show derivative plots of the hybridization curve for an mRNA population assumed to contain two (O), three (●), or four (×) frequency classes. (b) Derivative plots of the hybridization of total tumor polysomal [$^3$H]cDNA to its template mRNA (×), to weanling mRNA (●), and to normal mRNA (O). The corresponding curves are shown in Figure 2b.

solid circles in Figure 2b. Although the hybridization of tumor cDNA with RNA from regenerating liver of weanling rats is slower than the homologous reaction (solid line, tumor RNA × tumor cDNA), the heterologous curve has a saturation level which is identical with that of the homologous curve. This suggests that there are differences in the abundance of transcripts between hepatoma and regenerating liver but that the regenerating liver appears to contain most, if not all, of the sequences present in the tumor. It has been shown previously that the sequence complexity of polysomal mRNA from the regenerating liver of weanling rats is similar to that of normal liver (Scholla et al., 1980).

One useful way to compare multicomponent homologous and heterologous hybridization curves is to plot the derivatives of hybridization values against their respective $R_0t$ values. The components of the curves, which represent RNA frequency classes in the population, can then be easily visualized. These plots were described by Quinlan et al. (1978) and have been useful for analysis of hybridization data from cell cultures and regenerating liver (Siegal et al., 1979; N. Fausto and C. A. Scholla, unpublished experiments). The derivative plots permit the determination of the number of components (frequency classes) of a complex hybridization curve independently of the assumptions used for the computer fitting of the data. This is valid as long as the actual number of frequency classes in the population is equal to or smaller than the number of classes which are assumed to exist for curve fitting of the data points. Figure 3a illustrates the use of derivative plots for the calculation of the number of frequency classes in an mRNA population. The data obtained from the hybridization of tumor RNA with tumor cDNA have been plotted from computer calculations for which it was assumed that the mRNA population contained two, three, or four abundance classes. The figure shows that the sequences of this mRNA population can be best grouped into three abundance classes, because the number of peaks in the derivative plots ceases to increase (and remains as three) for calculations which assume that the population has more than three abundance classes.

Figure 3b presents the derivative plots of the hybridization curves of tumor cDNA with its own template RNA (homologous reaction) and with RNAs from normal and regenerating livers (curves shown in Figure 2b). The homologous curve differs more obviously from the curve of normal liver mRNA and is closest to the regenerating liver mRNA curve. The most significant differences are found in the first component of the curves ($R_0t < 10$), which corresponds to abundant mRNAs. Normal liver appears to lack, or has in much reduced frequency. RNAs which are abundant in the tumor; these differences are much less pronounced when tumor polysomal mRNA is compared with polysomal RNA from regenerating liver.

*Hybridization of Polyadenylated mRNA from Free Polysomes with cDNA.* It is logical to assume that neoplastic transformation should primarily affect nonsecreted proteins which participate in metabolic or growth processes within liver cells. Proteins which are not exported by the liver cells are generally synthesized in free polysomes. To examine more directly mRNAs from normal livers and hepatoma 252 which may code for these proteins, we isolated polyadenylated mRNA from free polysomes of normal liver and hepatoma. A comparison between the hybridization of polyadenylated mRNA of free polyribosomes of normal liver with its cDNA and the hybridization of mRNA of total liver polysomes with its homologous cDNA is shown in Figure 4a. The kinetics of the reactions suggest that, in free polysomes, the most abundant mRNA class represents a smaller proportion of the population.

To determine if free polysomes of the hepatoma lack mRNAs which are present in normal liver free polysomes or, conversely, if tumor free polysomes contain sequences not detected in normal liver, we did the following analyses (Figure 4b,c): (a) hybridization of cDNA transcribed from mRNA isolated from free polysomes of normal liver with polyadenylated mRNA obtained from free polysomes of the tumor (Figure 4b; for comparison, the hybridization of the same cDNA preparation with its template mRNA obtained from free polysomes of normal liver is also shown); (b) hybridization of cDNA transcribed from mRNA obtained from free polysomes of the hepatoma with mRNA isolated from free polysomes of normal livers (Figure 4c; for comparison, the hybridization of the same cDNA preparation with its template RNA from free polysomes of the tumor is also presented). The

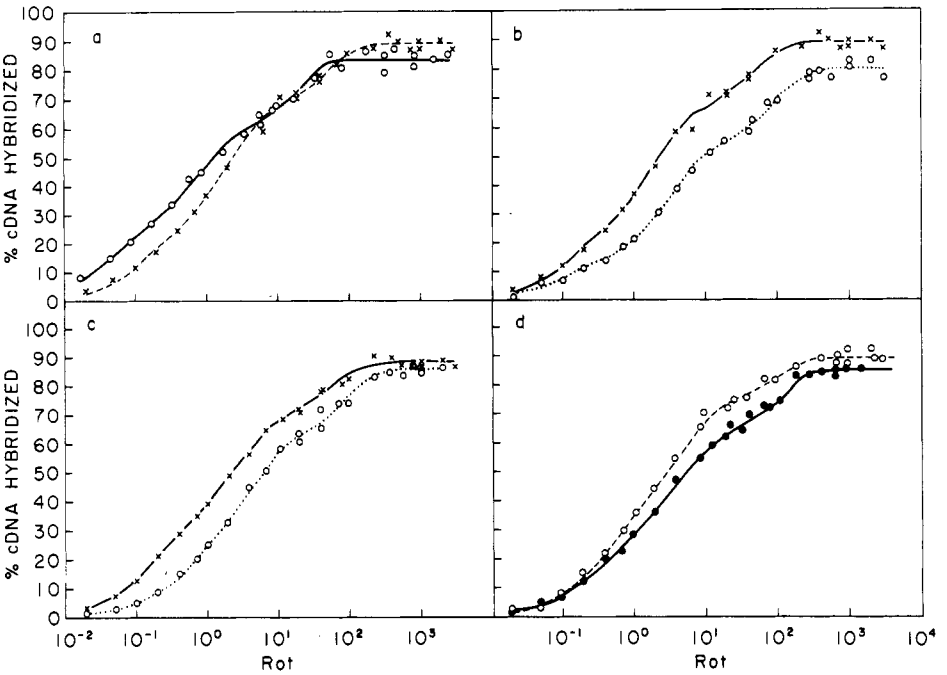


FIGURE 4: Hybridization of poly(A)$^+$ mRNA isolated from free polysomes of normal, regenerating liver and hepatoma. See Experimental Procedures and the legend to Figure 2 for hybridization conditions. (a) Hybridization of normal liver mRNA isolated from total polysomes to its complementary [$^3$H]cDNA (O); hybridization of normal liver mRNA isolated from free polysomes to its complementary [$^3$H]cDNA (×). (b) Hybridization of free polysomal normal [$^3$H]cDNA to polysomal poly(A)$^+$ mRNA isolated from free polysomes of hepatoma 252 (O) and to its template mRNA (×). (c) Hybridization of free polysomal tumor [$^3$H]cDNA to polysomal poly(A)$^+$ mRNA isolated from free polysomes of normal livers (O) and to its template mRNA (×). (d) Hybridization of cDNA transcribed from poly(A)$^+$ mRNA from free polysomes of regenerating liver (24 h after partial hepatectomy) to poly(A)$^+$ mRNA of free polysomes of hepatoma 252 (●) and to its template mRNA (O).

curves shown in Figure 4b indicate that ~10% of the mass of mRNA found in free polysomes of normal liver is absent from free polysomes of the hepatoma. The heterologous hybridization curve for mRNA from tumor free polysomes shown in Figure 4b differs markedly from the corresponding homologous curve in its kinetics and saturation level, although the differences between these curves at the very low $R_0t$ values are less pronounced than those of the curves for tumor total polysomal mRNA presented in Figure 2a. It is also important to determine if free polysomes of hepatoma 252 contain mRNA sequences which are not detected in normal liver free polysomes. The data regarding this question are presented in Figure 4c. The saturation levels of the homologous and heterologous curves overlap (88 ± 1.91 for the homologous reaction and 86 ± 1.17 for the heterologous curve), an indication that the tumor probably does not contain sequences which are absent from normal livers. However, these curves suggest that some sequences which are shared by both tumor and normal liver free polysomes are more abundant in the tumor.

A computer analysis of the homologous curves presented in Figure 4b,c is shown in Table I. For this analysis, the mRNA populations of free polysomes of normal liver and tumor were assumed to be distributed into three frequency classes (see below). The complexity of these mRNA populations is approximately 16 000 sequences, a value which is similar to our previous estimates of the sequence complexity of polysomal mRNA from normal and regenerating liver (Colbert et al., 1977; Scholla et al., 1980). Despite the similarity in sequence complexity of mRNA from free polysomes of normal liver and tumor, the data suggest that the hepatoma mRNA sequences which constitute the first frequency class (highly abundant mRNAs, present in an average number of ~5000 copies/cell) differ from the mRNA sequences found in the highly abundant class in normal liver. This conclusion

Table I: Analysis of Hybridization Kinetics and Determination of Sequence Complexity

| hybridization | component | % cDNA hybridized[a] | $R_0t$(cor)[b] | RNA sequence complexity[c] |
|---|---|---|---|---|
| normal RNA × normal cDNA[d] | I | 21.2 | 0.021 | 35 |
| | II | 49.0 | 0.72 | 1 169 |
| | III | 29.8 | 9.92 | 16 000 |
| tumor RNA × tumor cDNA[e] | I | 31.2 | 0.041 | 66 |
| | II | 43.2 | 0.84 | 1 361 |
| | III | 25.6 | 10.22 | 16 487 |
| tumor RNA × normal cDNA[f] | I | 12.9 | | |
| | II | 48.3 | | |
| | III | 38.8 | | |

[a] Normalized to 100% hybridization. [b] Corrected $R_0t_{1/2}$ = $R_0t_{1/2}$ of component class if that class were the only one present in the population. [c] Sequence complexity is the number of different sequences of average nucleotide length in the population. Mouse globin mRNA was used as a standard (Colbert et al., 19⁻⁻; Scholla et al., 1980). [d] Hybridization of mRNA from free polysomes of normal liver with its homologous cDNA [data from Figure 4b (×)]. [e] Hybridization of mRNA from free polysomes of hepatoma 252 with its homologous cDNA [data from Figure 4c (×)]. [f] Hybridization of mRNA from free polysomes of tumor with cDNA transcribed from mRNA of free polysomes of normal liver [data from Figure 4b (O)].

is suggested by the comparison between the percentage of cDNA which hybridizes with mRNA in the two hybridization reactions driven by tumor mRNA shown in Table I. When hybridized against its homologous cDNA, the first frequency class in tumor mRNA corresponds to 31% of the cDNA. However, when the same mRNA is hybridized against normal liver cDNA, the first RNA frequency class corresponds to 13% of the cDNA. These results suggest that mRNAs which are highly abundant in the normal liver are either lacking or found in lower frequency in the hepatoma, in agreement with the

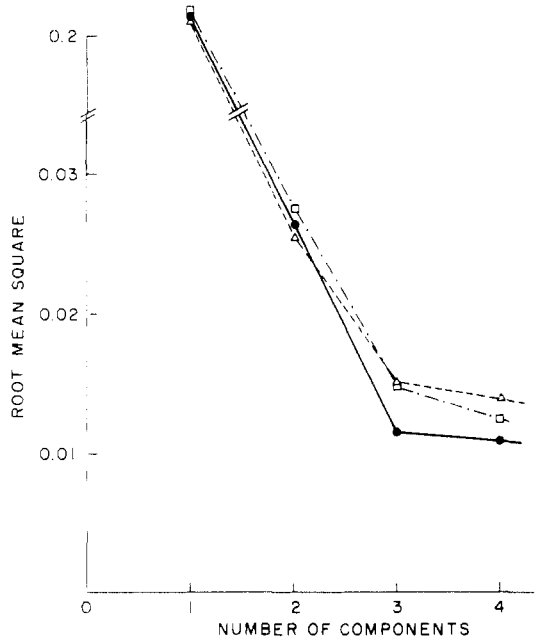


FIGURE 5: Determination of the number of frequency classes in mRNA populations of normal, regenerative liver and hepatoma 252. Relationship between the number of components assumed to exist in each population (abscissa) and the root mean square of the computer fitting for hybridization curves. Hybridization of cDNA transcribed from free polysomal mRNA of normal liver with its template mRNA [(△); curve shown in Figure 4b, solid line]; hybridization of cDNA transcribed from free polysomal mRNA of regenerating liver with its template mRNA [(□); curve shown in Figure 4d, dashed line]; hybridization of cDNA transcribed from mRNA of tumor free polysomes with its template mRNA [(●); curve shown in Figure 4c, solid line]. For each mRNA population analyzed, the same data points were used for a successive series of nonlinear least-squares analyses in which the population was assumed to contain one, two, three, or four abundance classes.

results of Reiners & Busch (1980).

Reiners & Busch (1980) concluded that the sequence complexity of Novikoff ascites hepatoma cytoplasmic mRNA is ~3 times higher than that of cytoplasmic mRNA of normal or regenerating liver. They also indicated that the mRNA population of the tumor can be best divided into four frequency classes while normal or regenerating liver mRNA is distributed into three frequency components. Presumably, sequences which were detected only in the ascites hepatoma were primarily found in the fourth abundance class, which contained ~5 copies/cell. The decision as to the number of frequency classes of an mRNA population depends on computer analysis of the hybridization curves and is often somewhat arbitrary. The curves should be analyzed as if they contained two, three, four, or more frequency classes, and a decision as to the optimal number of classes present in the population is made by plotting the computer fitting for the data points against the number of components which were assumed to exist in the population (Hastie & Bishop, 1976; C. A. Scholla and N. Fausto, unpublished experiments). This type of plot is shown in Figure 5 for data which correspond to the hybridization of mRNA from free polysomes of normal, regenerating liver and hepatoma 252 against their homologous cDNA. The fitting of the data points improves considerably as the number of RNA abundance classes assumed to exist increases. However, only a minimal improvement in the fitting takes place if the number of frequency classes assumed to exist changes from three to four. Thus, these mRNA populations can be assumed to contain mRNA sequences distributed into either three or four abundance classes, but, more importantly, the number of RNA abundance classes appears to be the same in mRNA populations of normal, regenerating liver and hepatoma.

*Comparison between Polyadenylated mRNA from Hepatoma 252 and Regenerating Liver.* The data presented in Figure 2b show that regenerating liver mRNA extracted from total polysomes probably contains most of the sequences present in mRNA of total tumor polysomes, although in different frequencies. The same conclusions also apply to polyadenylated mRNA populations from free polysomes as indicated by the results of experiments in which cDNA transcribed from tumor free polysomal mRNA (same cDNA preparation as in Figure 4c) was hybridized with free polysomal mRNA from regenerating livers (data not shown). To determine if the mRNA population of free polysomes of the hepatoma lacks sequences which might be found in regenerating liver, we hybridized hepatoma mRNA (from free polysomes) with cDNA synthesized from mRNA of 24-h regenerating liver free polysomes. A comparison between the curves shown in Figure 4d indicates that the hepatoma mRNA does not contain or has in much lower frequency mRNA sequences which are present in free polysomes of regenerating liver. It should be noted that the differences between mRNA populations of tumor and regenerating liver are less pronounced than those between tumor and normal liver mRNA.

## Discussion

We attempted to determine if total or free polysomes of hepatoma 252 lack mRNAs which are present in normal liver and if these polysomes might contain specific sequences not found in normal or regenerating livers. Our data show that mRNA obtained from total polysomes of hepatoma 252 lacks sequences which, on a mass basis, correspond to ~10% of the normal liver total polysomal mRNA. The absence of normal liver sequences in RNAs obtained from transplantable hepatomas has been previously reported (Tsé et al., 1978; Knöchel et al., 1980; Reiners & Busch, 1980). In Novikoff hepatoma, Reiners & Busch (1980) have shown by using fractionated cDNAs that the sequences which are missing in the hepatoma belong to normal liver abundant mRNAs. We show in this paper that mRNA sequences which are very abundant in normal liver free polysomes are missing or found only in lower concentrations in tumor-free polysomes. The proteins which are coded for by these mRNAs are not known at this time. However, one may speculate that the missing mRNAs code for nonexportable liver proteins which are abundant in normal liver. It remains to be established if these proteins are in any way directly related to neoplastic transformation in rat liver. Our results also suggest that free polysomes of hepatoma 252 do not contain, or have in reduced frequency, polyadenylated mRNA sequences which are present in regenerating liver.

Although, with the techniques used in this work, we showed that hepatoma 252 lacks normal liver mRNA sequences, we did not obtain unequivocal data which would suggest that the hepatoma contains specific mRNA sequences not detected in normal or regenerating liver. A comparison between free polysomal mRNA of tumor and normal liver suggested that there are obvious quantitative differences in the distribution of sequences between these two mRNA populations. However, it is doubtful that tumor mRNA contains a substantial number of "new" sequences which are lacking in normal or regenerating liver polysomes. In their analysis of mRNA from Novikoff hepatoma, Reiners & Busch (1980) concluded that tumor cytoplasmic mRNA contained several thousand mRNA sequences not present in regenerating livers. The tumor-specific mRNA constituted a fourth frequency class of high-complexity sequences not detected in regenerating livers. In our studies, we did not detect an additional high-complexity

frequency class in free or total polysomal polyadenylated mRNA of hepatoma 252. We also found that the computer-estimated sequence complexity of polyadenylated mRNA from free polysomes of this tumor is not different from that of mRNA from free polysomes of normal liver. The analysis of the derivative plots of homologous and heterologous reactions suggests that the tumor contains, in lower concentration, mRNA sequences which are abundant in normal or regenerating livers and that, conversely, some sequences are more abundant in the hepatoma than in normal livers. The results are in complete agreement with the work of Jacobs & Birnie (1980) and that of Capetanaki & Alonso (1980). Working with a minimal-deviation rat hepatoma cell line, Jacobs & Birnie (1980) found by analyzing the hybridization kinetics of polysomal mRNA of the tumor cells that few sequences are specific to these cells and that polysomes of this hepatoma line contain, in greatly reduced frequency, mRNA sequences which are abundant in normal livers. Work with other cell systems in culture, including the studies of Moyzis et al. (1980) using a highly tumorigenic cell line, has also not demonstrated the existence of cellular mRNA sequences which are specific for the transformed state (Getz et al., 1977; Rolton et al., 1977; Williams et al., 1977).

The differing results of analyses of hepatoma mRNA reported in the literature could probably be explained by the variable biological characteristics of these tumors. For instance, hepatoma 7777 which apparently contains sequences not found in normal liver synthesizes $\alpha$-fetoprotein in large amounts but has a decreased rate of albumin synthesis (Tsê et al., 1978; Sala-Trepat et al., 1979). Other rat hepatocellular carcinomas contain these two proteins in various ratios while hepatoma 252 does not appear to contain either albumin or $\alpha$-fetoprotein mRNAs (Sell et al., 1979). In addition, comparisons between polysomal mRNAs of hepatomas and normal or regenerating livers may yield different results from work using mRNA from whole-cell or cytoplasmic extracts.

The conclusions reached in this report are limited by a variety of uncertainties regarding the methodology which is generally used in these studies. For instance, although it is assumed that cDNA transcribed from a template composed of a complex mixture of RNA molecules accurately reflects the compositon of that RNA population, there is no definitive proof that this is indeed the case. Moreover, the sensitivity of the hybridization reactions is not great enough to detect very small differences in complexity between two RNA populations. Nevertheless, in this work, losses of sequences and decreases in sequence abundance in tumor mRNA preparations were easily detected. In contrast, there was little indication for the existence of a substantial fraction of tumor-specific sequences. If these specific sequences exist, they must constitute only a very small fraction of the polysomal RNA mass of hepatoma 252. In any event, in polysomal mRNA populations of this tumor, qualitative changes which represent genetic restriction are much more conspicuous than alterations which are the result of derepression. It is of interest that in this hepatoma, free polysomes, which presumably contain mRNA which codes for nonexportable proteins, lack a considerable amount of RNA present in normal or regenerating livers.

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