Transcriptional and Posttranscriptional Modulation of Cytoplasmic Ribonucleic Acids in Regenerating Liver and Novikoff Hepatoma†

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ABSTRACT: Complementary deoxyribonucleic acid (cDNA) was synthesized to cytoplasmic poly(A^+) ribonucleic acids (RNAs) of 18-h regenerating rat liver and Novikoff hepatoma ascites cells to generate probes for comparison of cytoplasmic RNAs. Approximately 94% of the tumor cDNA formed hybrids with regenerating liver cytoplasmic poly(A^+) RNA; however, even at an R_0t value 10-fold greater than the maximum homologous reaction, only 72% of the unfractionated regenerating liver cDNA formed hybrids with tumor cytoplasmic poly(A^+) RNA. Hybridization analyses with regenerating liver cDNAs separated into populations representing abundant and less abundant frequency RNAs demonstrated that the tumor contained most of the regenerating liver cytoplasmic sequence complexity and that the unhybridized cDNA represented a low complexity, abundant frequency RNA class. The less abundant frequency RNAs were present in the cytoplasm and nuclei of both cell types in roughly equivalent copy numbers. However, the abundant frequency regenerating liver sequences were 200–10 000 times more abundant in the cytoplasm of regenerating liver than in the tumor. These abundant frequency sequences were also 100–1000-fold more abundant in regenerating liver nuclei. Sequence copy numbers were estimated and used to compare the cytoplasmic and nuclear contents of the different frequency RNAs. Relative to the nuclear content of the sequences represented by the regenerating liver abundant and less abundant frequency cDNAs, there was a 14-fold accumulation of abundant frequency RNAs in the cytoplasm of regenerating liver. An analogous accumulation of regenerating liver characteristic abundant frequency cytoplasmic RNAs did not occur in the tumor. These data suggest that transcriptional and possibly posttranscriptional mechanisms are responsible for the relative absence in the Novikoff hepatoma of a low complexity, abundant frequency liver RNA population.

Neoplastic transformation of normal cells is a complex and poorly understood process. The disappearance of normal cellular proteins and the appearance of new proteins are often consequences of transformation. Proteins such as serine dehydrase (Auerback & Waisman, 1958), tryptophan oxygenase (Feigelson et al., 1975), albumin (Schreiber et al., 1969; Ove et al., 1972; Sell, 1974; McLaughlin & Pitot, 1976), and α_{2U}-globulin (Sippel et al., 1976) are often absent or in reduced concentrations in hepatomas of varying degrees of malignancy. Casein levels are often significantly lower in rat mammary carcinomas (Rosen & Barker, 1976). The molecular basis for the depressed cellular contents of albumin, α_{2U}-globulin, and casein has been correlated with decreased cytoplasmic mRNA levels (Tse et al., 1978; Sala-Trepat et al., 1979; Sippel et al., 1976; Rosen & Barker, 1976; Rosen & Socher, 1977).

The cytoplasmic contents of specific mRNAs could be determined by either transcriptional or posttranscriptional modulation. Regulation of the rate of transcription has been postulated as the primary mechanism for controlling mRNA content (Davidson & Britten, 1973) and is certainly important for genes under hormonal control (Kurtz & Feigelson, 1977; Monahan et al., 1976; Palmiter et al., 1976; Hynes et al., 1977). However, differential processing and transport of nuclear transcripts could also determine cytoplasmic sequence abundance. In addition, differential stabilization or degradation of nuclear and cytoplasmic RNAs could result in the enrichment of classes of RNAs (Kafatos, 1972). A correlation between cytoplasmic RNA abundancy and stability has been demonstrated for the abundant cytoplasmic sequences of mouse L cells (Meyuhas & Perry, 1979) and *Drosophila* cells (Lenk et al., 1978).

Previous investigations from this laboratory have demonstrated that 97% of the detectable cytoplasmic and nuclear proteins of the Novikoff hepatoma ascites cell and normal and regenerating rat liver are mutually common and in similar quantities (Hirsch et al., 1978a; Takami & Busch, 1979; Takami et al., 1979). Two-dimensional analyses of regenerating liver and tumor in vitro translation products synthesized from unfractionated mRNAs and mRNAs chromatographed on cDNA–cellulose columns suggested that there were a limited number of abundant regenerating liver sequences which are much less abundant in the tumor (Hirsch et al., 1978b).

In this study, cDNA–RNA hybridization analyses were used to define cytoplasmic RNA populations which are either exclusively abundant in regenerating liver or mutually common and similarly abundant in regenerating liver and the Novikoff hepatoma. cDNAs representing these two RNA populations were used to estimate the cytoplasmic poly(A^+)[1] RNA and nuclear poly(A^+) and poly(A^-) RNA contents of these sequences in both tissues. By comparing the cytoplasmic to nuclear contents of these sequences, it was possible to determine whether transcription or posttranscriptional mechanisms were responsible for cytoplasmic messenger contents and the basis for the absence of regenerating liver abundant frequency cytoplasmic mRNAs in the tumor cytoplasm.

Experimental Procedures

Animals and Tumor Cells. Male Holtzman rats weighing 220–260 g were used in all studies. Novikoff hepatoma ascites cells were transplanted 6 days prior to sacrifice. Regenerating livers were removed 18 h after partial hepatectomy.

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[1] Abbreviations used: poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate, Tris, tris(hydroxymethyl)aminomethane; PB, equimolar Na_2HPO_4 and NaH_2PO_4; SSC, 0.15 M sodium chloride and 0.015 M sodium citrate; R_0t, moles of RNA nucleotides per liter times seconds; RNP, ribonucleoprotein.

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Preparation of Cytoplasmic RNA. Ascites cells were filtered through cheesecloth, pelleted by centrifugation, and washed twice in 10 mM Tris-HCl (pH 7.4)–10 mM NaCl–5 mM $MgCl_2$ (RSB). The washed cells were suspended in 10 volumes of RSB containing 50 μg/mL heparin, and Nonidet NP-40 was added to a final concentration of 0.75%. The lysed cells were homogenized with a Tekmar tissumizer (4 × 15 s, set 7) to reduce clumping, and the suspension was centrifuged for 5 min at 1500*g* to remove nuclei and cell debris. The supernatant fluid was further centrifuged for 15 min at 15000*g* and used as a source of cytoplasmic RNA.

The procedure of Taylor & Schimke (1974) was used with minor modifications for isolation of regenerating rat liver cytoplasmic RNA. Livers were perfused with cold 0.025 M Tris-HCl (pH 7.5)–0.025 M KCl–0.005 M $MgCl_2$ (TKM), excised, passed through a tissue press, suspended in 4 volumes of TKM containing 0.25 M sucrose and 50 μg/mL heparin, and homogenized in a Teflon–glass Potter–Elvehjem grinder. Triton X-100 was added to a final concentration of 1%, and the homogenate was centrifuged for 5 min at 1500*g* to sediment nuclei. The supernatant fluid was adjusted with a 20% deoxycholate solution to a final concentration of 1%, incubated for 5 min at 4 °C, and centrifuged for 15 min at 15000*g*.

Cytoplasmic RNA present in the 15000*g* supernatant fluids was isolated by Mg^{2+} precipitation (Palmiter, 1974). This technique precipitates both polysomes and cytoplasmic RNPs. The pellet obtained by Mg^{2+} precipitation was dissolved in 0.5% $NaDodSO_4$–10 mM Tris-HCl (pH 7.5)–0.1 M NaCl–0.005 M EDTA, centrifuged at 10000*g* for 10 min to remove insoluble material, and adjusted with the above solution to 15–20 A_{260} units/mL. RNA was deproteinized with phenol–chloroform as described by Palmiter (1974).

Preparation of Nuclei and Nuclear RNA. Nuclei were prepared and purified by several procedures. Nuclei and cell debris sedimented by centrifugation for 5 min at 1500*g* were resuspended in 2.2 M sucrose–10 mM $MgCl_2$–10 mM Tris (pH 7.5) (8 volumes/g of original tissue), homogenized in a loose-fitting Potter–Elvehjem grinder, filtered through cheesecloth, and centrifuged for 2.5 h at 15000*g*. Pelleted nuclei were resuspended in 0.88 M sucrose–10 mM $MgCl_2$ and recentrifuged. Alternately, nuclei were prepared by the citric acid method described by Taylor et al. (1973) as modified by Sippel et al. (1977). Rat liver, ascites cells, or nuclei sedimented by centrifugation for 5 min at 1500*g* were homogenized with a Tekmar tissumizer at 2 °C in 5 volumes (5 volumes/g of original tissue) of 2.5% citric acid–0.14% Triton X-100. The homogenate was filtered through cheesecloth and centrifuged for 5 min at 1500*g*. The pellet was washed once in the same solution and resuspended in 0.25 M sucrose–2.5% citric acid, and the nuclei were pelleted through a cushion of 0.88 M sucrose–2.5% citric acid by centrifugation for 10 min at 1500*g*. Tumor nuclei free of cytoplasmic tags were only obtained by the citric acid procedure. Nuclei were lysed by the addition of 15 volumes of 4% Sarkosyl–10 mM Tris-HCl (pH 7.5)–10 mM EDTA and either directly deproteinized by phenol–chloroform extraction or centrifuged for 18 h at 200000*g* at 0 °C through a 10-mL pad of the above solution containing 20% sucrose. The resulting supernatant fluid was deproteinized by phenol–chloroform extraction and adjusted to 0.24 M ammonium acetate, and RNA was precipitated by the addition of 2 volumes of ethanol. Precipitated nuclear RNA was dissolved in 0.01 M Tris-HCl (pH 7.5)–0.01 M NaCl–0.005 M $MgCl_2$, adjusted to 15–20 A_{260}/mL, and treated for 30 min at 26 °C with RNase-free DNase to remove residual DNA (1 mg of DNase per 300 A_{260}). The reaction was terminated by adjusting the solution to 2 mM EDTA, 0.5% Sarkosyl, and 50 mM sodium acetate, pH 5.1. The RNA was deproteinized by two extractions with chloroform and precipitated with ammonium acetate and ethanol.

Isolation of Poly(A) RNA. Precipitated cytoplasmic or nuclear RNA was dissolved in 0.5% $NaDodSO_4$–10 mM Tris (pH 7.5)–400 mM NaCl, adjusted to 10–15 A_{260}/mL, denatured by heating to 65 °C for 5 min, quick-chilled, and chromatographed on a column of oligo(dT)–cellulose. The unbound RNA was recycled through the column, and the column was washed with the above buffer. Bound RNA was eluted with 10 mM Tris-HCl (pH 7.5)–0.2% $NaDodSO_4$ and readjusted to 0.4 M NaCl, and the entire procedure was repeated twice. RNA was precipitated by the addition of ammonium acetate to 0.24 M and 2 volumes of ethanol.

Synthesis and Fractionation of cDNA. cDNAs were synthesized and isolated by the procedure of Samal & Bekhor (1979) with the following modifications: no pyrophosphate was included; the DTT concentration was lowered to 4 mM; 15–20 units of reverse transcriptase was used per 0.1 mL; synthesis was allowed to proceed for 30 min. All cDNAs (1.0 × 10^7 cpm/μg) were dissolved in H_2O and stored at −20 °C.

Regenerating liver cDNA was hybridized with a 2600-fold excess of regenerating liver cytoplasmic poly(A^+) RNA to an R_0t of 0.8 M s. The reaction mixture (0.1 mL) was diluted 40-fold with 0.03 M sodium phosphate buffer, pH 6.8 (PB), and loaded onto a water-jacketed hydroxylapatite column equilibrated at 60 °C with 0.03 M PB. Unhybridized cDNA was eluted with 0.14 M PB. cDNAs existing in cDNA–RNA duplexes (representing abundant copy species) were eluted with 0.45 M PB. Eluted cDNA fractions were pooled, adjusted to 0.3 M NaOH, incubated for 30 min at 73 °C, neutralized, and chromatographed on a column of Sephadex G-50 equilibrated with 20 mM Tris-HCl, pH 7.5. The fractions containing [^{3}H]cDNA were pooled, and 100 μg/mL yeast tRNA was added as a carrier. The [^{3}H]cDNAs were precipitated by the addition of ammonium acetate and ethanol.

Hybridization Conditions. All RNA preparations were dissolved in H_2O and passed through small columns of neutralized Chelex 100 prior to hybridization. Hybridizations were performed at 69 °C in small polypropylene vials under mineral oil. The hybridization reaction mixture (10 μL) contained 1000–2000 cpm of [^{3}H]cDNA, RNA, 0.3 or 0.45 M NaCl, 1% $NaDodSO_4$, 2 mM EDTA (neutralized), 10 μg of yeast tRNA, and 10 mM Tris-HCl, pH 7.1 (at 69 °C). RNA was always in a large excess, and incubation times were limited to a maximum of 100 h. The reactions were terminated by immersion in an acetone–solid CO_2 bath, and the amount of [^{3}H]cDNA existing in hybrids was determined by resistance to S_1 nuclease from *Aspergillus oryzae* as described by Samal & Bekhor (1979). All hybridization data have been normalized to rates of reaction occurring at 0.12 M PB and are expressed as log equivalent R_0t. Data were analyzed by using a computer program (Monahan et al., 1977) designed to fit data according to the equation $C/C_0 = B + [1 - \exp(0.693R_0t)/(R_0t_{1/2})]$, where C/C_0 represents the fraction of [^{3}H]cDNA in hybrid form at time x, B is the "zero time" hybridization value, R_0t = mol s L^{-1} of RNA nucleotides, and $R_0t_{1/2}$ = mol s L^{-1} of RNA nucleotides at 50% hybridization.

Poly(A) concentrations were determined by titration with [^{3}H]poly(U). Hybridizations were performed for 16 h at 37 °C in reaction mixtures containing 2 × SSC, 10 mM Tris, pH 7.1, RNA, and [^{3}H]poly(U). Hybrid formation was assayed by resistance to S_1 nuclease or RNase as described by Bishop & Rosbash (1974).

Table I: Sizes of Regenerating Liver and Novikoff Hepatoma Cytoplasmic and Nuclear Poly(A$^+$) RNAs and Poly(A) Moieties

	number-average nucleotide length[a]			
	cytoplasm		nuclear	
source	RNA	poly(A) moiety	RNA	poly(A) moiety
Novikoff hepatoma	1200	120	1600	212
regenerating liver	1550	105	1900	260

[a] Average of three determinations.

Characterization of 3'-Terminal Poly(A). The nucleotide lengths of poly(A) in cytoplasmic and nuclear poly(A$^+$) RNAs were determined by a modification of the procedure of Morrison et al. (1973). RNA (2–4 μg) was dissolved in 25 μL of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 300 mM NaCl, 3.5 units of RNase A, and 1 unit of RNase T_1 and incubated for 1 h at 37 °C. The digest was subjected to electrophoresis on 98% formamide–polyacrylamide gels. The gels were sliced into 2-mm sections after electrophoresis and soaked in 200 μL of 2 × SSC for 48 h at room temperature. The size distribution of the poly(A) moieties was determined by hybridization of a sample of the eluate with [^{3}H]poly(U).

Polyacrylamide Gel Electrophoresis in 98% Formamide. The procedure of Duesberg & Vogt (1973), as described by Sippel et al. (1977), was used for sizing RNA, [^{3}H]cDNA, and poly(A) on polyacrylamide gels in 98% formamide. RNA and [^{3}H]cDNA were sized in 2.5 or 3.5% acrylamide gels. Cytoplasmic and nuclear poly(A) moieties were sized on 9 and 6.5% acrylamide gels, respectively. After electrophoresis, gels were sliced into 2-mm sections and RNA or poly(A) was eluted as described above and hybridized with [^{3}H]poly(U). Slices containing [^{3}H]cDNA were solubilized in 30% H_2O_2 and counted in Formula 947 (New England Nuclear). 28S, 18S, and 8S RNA, U_1 (169 nucleotides; Hellung-Larson, 1977; Reddy et al., 1974), 5.8S and 5S RNA, and tRNA were used to calibrate the gels and were identified by staining with methylene blue.

RNA and DNA Determinations. DNA and RNA were estimated by the procedures of Richards (1974) and Hurlbert et al. (1954), respectively.

Results

Isolation and Characterization of Poly(A$^+$) RNAs. Complexity estimates derived by cDNA–RNA hybridization analyses are dependent upon the concentration of the driver species. The concentration of a poly(A$^+$) RNA population can be accurately determined from the number-average nucleotide lengths of the RNA and poly(A) moiety. Given the size relationship between the poly(A) moiety and intact RNA species, estimates of purity can be determined by hybridization of [^{3}H]poly(U) to the poly(A) moiety. Table I shows that the number-average nucleotide length of the regenerating liver cytoplasmic poly(A$^+$) RNAs (1550) was larger than that of the tumor (1200). Our value for regenerating liver is similar to the estimate of 1500 nucleotides reported by Atryzek & Fausto (1979). The number-average nucleotide lengths of the cytoplasmic poly(A) moieties were similar for the two tissues and represented 6.8–10% of the total RNA length. Consequently, the average potential coding capacity of the tumor cytoplasmic RNAs was less than that of the liver. Tumor and liver nuclear poly(A$^+$) RNAs were slightly larger than their respective cytoplasmic counterparts; ~25% of the increased length was due to the greater length of the poly(A) moiety.

The amounts of Mg^{2+}-precipitated cytoplasmic RNA per cell and the percentages of nuclear RNA existing as polyadenylated molecules were similar for the two tissues (Table II). However, the ratios of DNA to nuclear RNA are 4.2 and 1.6 in regenerating liver and tumor nuclei, respectively. If 5.9 pg of DNA is used as an estimate of the diploid rat genome (Savage et al., 1978), then there are two copies of the diploid genome per rat liver cell. This agrees with the data of Gonzales & Mathias (1973), who showed that adult rat cells are mainly tetraploid. The increased amount of DNA in the tumor relative to the liver tissues may be indicative of the polyploidy and anueploidy of the tumor. The increased amount of RNA in the nucleus of the tumor could be due to differences in the rate of nuclear processing of pre-mRNAs and pre-rRNAs or could reflect the large amounts of rRNA being synthesized by the multiple copies of rDNA in the large tumor nucleoli.

Table II: Cellular Amounts of Poly(A$^+$) RNA in Cytoplasm and Nuclei of 18-h Regenerating Liver and the Novikoff Hepatoma[a]

	RNA/cell[b]				
	cytoplasm[c]		nuclei[d]		
source	pg	% poly(A$^+$) RNA[e]	pg	% poly(A$^+$) RNA[e]	DNA/cell (pg)
Novikoff hepatoma	48.8	1.93	14.0	10.5	22.2
regenerating liver	49.8	2.5	2.6	8	11

[a] Typically, 96–102% of the initial total cellular RNA was recovered in the post 15000*g* supernatant fluid and the nuclear pellet. DNA recoveries (92–100%) were used to correct for RNA losses during isolation of nuclei. [b] RNA contents were 6.75 and 9.15 mg of RNA per g of Novikoff hepatoma and 18-h regenerating liver, respectively. The values of 1.5×10^8 cells/g of regenerating liver (Sippel et al., 1977) and 1.097×10^8 cells/g of tumor were used to calculate RNA/cell. [c] Cytoplasmic values are for the amounts of RNA recovered in the Mg^{2+} precipitates. [d] Assumes 1 nucleus/cell. [e] Calculated from [^{3}H]poly(U) titrations.

Nuclear Contamination of Cytoplasmic RNA. Mg^{2+} precipitation was used for preparation of cytoplasmic mRNAs because polysome banding or pelleting procedures do not isolate monosomes or free mRNP, which constitute 30–40% of total rat liver cytoplasmic poly(A$^+$) RNA (Atryzek & Fausto, 1979). Mg^{2+} precipitation, however, also results in the coisolation of nuclear mRNPs. Several criteria suggest that nuclear breakage or leakage did not exceed 1–3% in our experiments. First, less than 1% of the total cellular DNA was detected in the post 15000*g* supernatant fluid. Second, the poly(A) moieties of cytoplasmic and nuclear RNAs were easily resolved by electrophoresis. Two to three percent of the cytoplasmic RNAs had a poly(A) moiety similar in length to the average nuclear poly(A) moiety. Third, RNA was isolated from hepatectomized rats and rats with tumor cells pulsed with [^{3}H]orotic acid for 10 min. One to two percent of the total labeled RNA greater than 32 S was cytoplasmic. By the assumption that the rapidly synthesized RNA greater than 32 S is preribosomal and nuclear (Sippel et al., 1977), nuclear breakage did not exceed 2%. From the data in Table II and an assumption of 3% nuclear breakage, it can be estimated that nuclear RNAs constituted at most 0.5 and 4.5% of the total cytoplasmic poly(A$^+$) RNAs of regenerating liver and the tumor, respectively.

Synthesis and Characterization of cDNAs. Comparisons of the sizes of the cDNAs and their respective cytoplasmic poly(A$^+$) RNA templates are shown in Figure 1. The profiles for cDNA and template RNA are essentially the same. The number-average nucleotide lengths of the RNA/cDNA for regenerating liver and tumor are 1550/1420 and 1200/1050,

Table III: Sequence Complexity of Cytoplasmic Poly(A$^+$) RNAs[a]

source	frequency class	proportion of cDNA hybridized (%)	obsd $R_0t_{1/2}$ (M s)	cor $R_0t_{1/2}$[b] (M s)	complexity[c] (daltons)	no. of RNA species[d]	copies/cell[e]
regenerating liver	I	30.8	0.115	0.035	2.4×10^7	47	9800
	II	27.8	1.40	0.39	2.6×10^8	519	800
	III	41.4	24.5	10.14	6.8×10^9	13550	45
Novikoff hepatoma	I	15.8	0.04	0.0063	3.7×10^6	9	25600
	II	32.2	0.8	0.25	15.1×10^7	386	1200
	III	37.2	12.0	4.46	26.3×10^8	6737	80
	IV	13.8	180	24.84	15.4×10^9	37460	5

[a] Analyses of hybridization data in Figure 2. The computer-estimated 95% confidence limit for R_0t estimates was observed $R_0t \pm 4$–7%. [b] Observed $R_0t_{1/2}$ values were corrected by multiplication with the proportion of cDNA hybridized. [c] Complexities were calculated from the corrected $R_0t_{1/2}$ values by using rabbit globin α and β mRNAs as a standard. Globin RNA of 1200 nucleotides had a $R_0t_{1/2}$ of 9.52×10^{-4} when hybridized to a cDNA of 500–600 nucleotides. The rate of the standard reaction was corrected for the differences in cDNA size between the experimental cDNAs and the standard by assuming that the rate is proportional to the square root of the length of the cDNA (Hutton & Wetmur, 1973). [d] The number of RNA species was calculated by dividing the complexity estimate by the number-average nucleotide length of the RNA (Table I) and the average molecular weight of a nucleotide (325). [e] Copies of RNA per cell were determined as follows: [[poly(A$^+$) RNA/cell] × (fraction of cDNA hybridized) × (6×10^{23})]/(total complexity of RNA class).

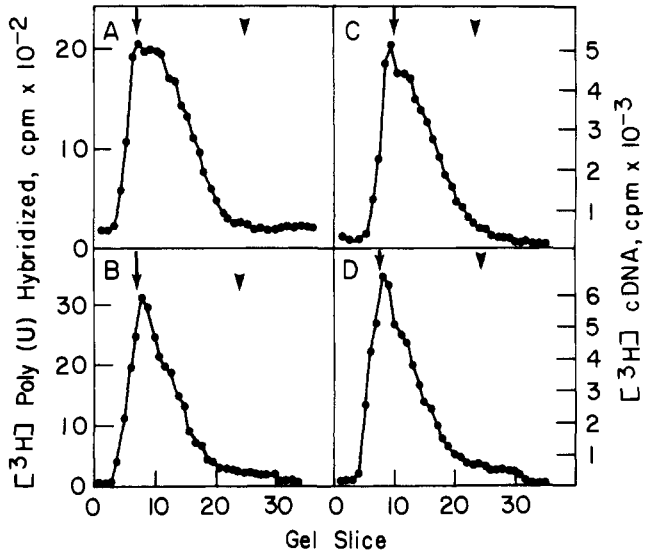

FIGURE 1: Size distribution of template cytoplasmic poly(A$^+$) RNAs and cDNAs on polyacrylamide gels in 98% formamide. Tumor (A and C) and regenerating liver (B and D) RNAs and cDNAs were analyzed on 3.5% acrylamide–98% formamide gels as described under Experimental Procedures. After electrophoresis, gels were sliced into 2-mm sections and the positions of RNAs were determined by titration with [^{3}H]poly(U) (panels A and B). Gel sections containing cDNAs (panels C and D) were solubilized in 30% H_2O_2 and counted as described under Experimental Procedures. The positions of 18S RNA (↓) and 5.8S RNA (▼) were determined in parallel gels by staining with methylene blue.

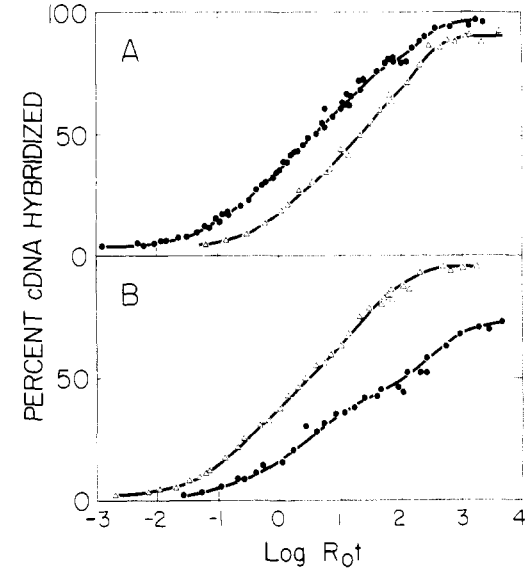

FIGURE 2: Homologous and heterologous kinetics of hybridization of cDNAs with Novikoff hepatoma and regenerating liver cytoplasmic poly(A$^+$) RNAs. Regenerating liver RNA (△) and tumor RNA (●) were hybridized to tumor cDNA (A) and regenerating liver cDNA (B) as described under Experimental Procedures. The R_0t values were obtained by varying the concentration of RNA (0.04–2000 μg/mL) and the duration of incubation (up to 100 h). All hybrid reactions represent data obtained with two or more preparations of RNA.

respectively. Considering that the cDNA synthesis reaction is primed with an oligomeric chain of 12–18 residues, the differences between cDNAs and their respective templates are probably due to incomplete transcription of the 3′-poly(A) moiety. The cDNAs were 1% double stranded as measured by resistance to S_1 nuclease. These data suggest that the cDNAs are almost full-length transcripts and represent the diversity and relative proportion of individual RNA species within the template population.

Hybridization of cDNA to Cytoplasmic Template RNA. The kinetics of hybridization for tumor and regenerating liver cDNAs with their cytoplasmic RNA templates are shown in parts A and B of Figure 2, respectively. In each preparation, 95–98% of the cDNAs formed S_1 nuclease resistant hybrids with template RNA. The kinetics of hybridization extend over 5 orders of magnitude of R_0t and suggest the existence of a heterogeneous RNA population containing different frequency classes. The complexity of an unknown RNA population can be obtained by comparison of its hybridization kinetics to those of a known RNA. Under conditions of RNA excess (Hastie & Bishop, 1976), the rate of hybridization of RNA to cDNA is proportional to the base sequence complexity of the RNA.

The program used for complexity analysis fitted the data to a two-, three-, or four-component model based upon the relative abundancy of the RNAs. Regenerating liver cytoplasmic poly(A$^+$) RNAs were best fit by a three-component model (Table III). The present estimate of 14 100 RNA species is very similar to a previous report (Colbert et al., 1977).

The tumor cytoplasmic hybridization data fit best to a four-component model (Table III). Primarily because of the fourth component, tumor complexity was estimated to be 44 600 species. If a three-component model was assumed, the tumor complexity was 40 700 RNA species. (Reiners, unpublished experiments). The fourth frequency class represented 13.8% of the hybridizable tumor cDNA. We cannot eliminate the possibility that the putative nuclear contamination of cytoplasmic poly(A$^+$) RNA represents nuclear sparse

Table IV: Frequency Distribution of Abundant and Less Abundant Regenerating Liver Cytoplasmic Poly(A$^+$) RNAs in Regenerating Liver and Novikoff Hepatoma Cytoplasmic RNA[a]

source	frequency class	obsd $R_0t_{1/2}$ (M s)	cor $R_0t_{1/2}$[b] (M s)	total complexity (daltons)	no. of RNA species	copies/cell[c]
regenerating liver	abundant	0.08	0.03	1.98×10^7	39	15 500
	less abundant	17	10.79	7.26×10^9	14 500	66
Novikoff hepatoma	abundant	65			39	13
	less abundant	13			14 500	64

[a] The table is an analysis of the data in Figure 3. Observed $R_0t_{1/2}$ values for less abundant frequency RNAs were calculated from the data in Figure 3 which were plotted as separate curves in Figure 5. [b] Abundant and less abundant frequency cDNAs represent 36.5 and 63.5% of the hybridizable cDNAs, respectively. [c] The values for regenerating liver were calculated as described in Table III. The tumor RNA copy numbers were calculated as follows: [(liver corrected $R_0t_{1/2}$/observed tumor $R_0t_{1/2}$) × [cytoplasmic poly(A$^+$) RNA/cell] × (6×10^{23})]/(total complexity of RNA class).

copy sequences and is contained in this 13.8%. Furthermore, a 1% variation in the estimation of the $R_0t_{1/2}$ of this class alters the total sequence complexity by 1% (400 species). These results emphasize the limitations of cDNA–RNA hybridization analyses for estimation of complexity. Consequently, only relative estimates and comparisons of total sequence complexities can be made.

Estimates of the copies per cell of each RNA species within a frequency class were calculated from the hybridization data and yields of RNA per cell (Table III). Abundant frequency RNAs were present in ~10 000–25 000 copies/cell in contrast to less abundant RNAs which were present in 5–45 copies/cell. The data suggest that 48–58% of the cytoplasmic poly(A$^+$) RNA mass in both tissues represents only 1–4% of the total cytoplasmic poly(A$^+$) RNA sequence complexity.

Heterologous RNA–cDNA Hybridizations. Heterologous hybridizations were used to compare cytoplasmic poly(A$^+$) RNA populations. Regenerating liver cytoplasmic RNA hybridized to tumor cDNA four- to fivefold slower than template RNA (Figure 2A). However, by R_0t = 2000, regenerating liver RNA formed S_1 nuclease resistant hybrids with 94% of the tumor hybridizable cDNA. These data suggest that regenerating liver cytoplasmic RNAs contain RNA species which constitute 94% of the tumor's cytoplasmic poly(A$^+$) RNA mass. By the assumption that the 6% of nonhybridizable tumor cDNA is representative of the fourth tumor RNA frequency class, this small percentage represents ~16 300 RNA species and suggests that the regenerating liver shares 64% of the tumor complexity.

The hybridization kinetics of tumor cytoplasmic poly(A$^+$) RNA with regenerating liver cDNA are in marked contrast to the results of the reciprocal hybridization (parts A and B of Figure 2, respectively). The tumor hybridization curve was displaced 1–2 log R_0t units to the right of the homologous cDNA–RNA reaction. By R_0t = 3000, only 72% of the cDNAs had been hybridized.

Hybridization with Abundant and Less Abundant Frequency cDNAs. Hybridization analyses with unfractionated regenerating liver cDNAs suggested that the tumor lacked a high complexity, low frequency RNA class and/or a low complexity, high frequency regenerating liver RNA class. Regenerating liver cDNAs were hybridized with template RNA to an R_0t of 0.8 and separated by hydroxylapatite chromatography into cDNA populations representing abundant and less abundant RNA species to differentiate these possibilities. At this R_0t value, 36.5% of the hybridizable cDNA existed as a duplex. The cDNA fraction representing abundant frequency RNA species hybridized to regenerating liver RNA with an $R_0t_{1/2}$ of 0.08 and represents 39 RNA species (Figure 3A, Table IV). The cDNA fraction representing less abundant frequency RNA species hybridized to its template RNA with an R_0t of 17 and represents less

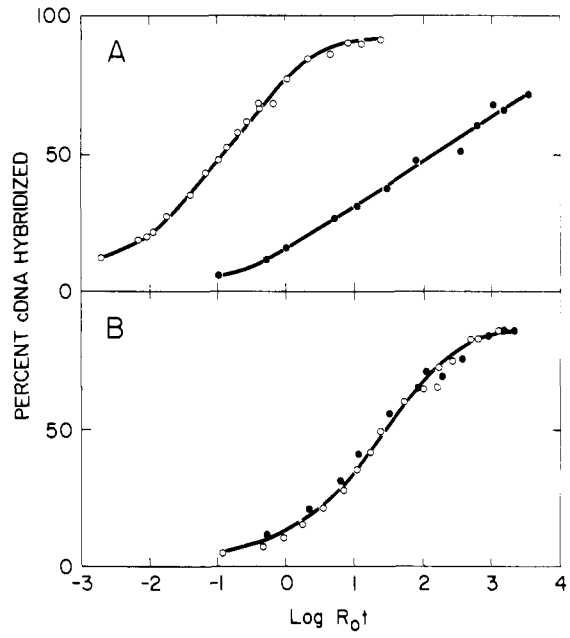

FIGURE 3: Hybridizations between regenerating liver abundant frequency or less abundant frequency cDNAs with regenerating liver and tumor cytoplasmic poly(A$^+$) RNAs. Abundant frequency cDNA (A) or less abundant frequency cDNA (B) was hybridized with regenerating liver (○) or tumor (●) cytoplasmic poly(A$^+$) RNA as described under Experimental Procedures.

abundant RNA species (Figure 3B, Table IV). The combined sequence complexity of 14 500 RNA species determined with the fractionated cDNAs agrees very well with the 14 100 species determined with the unseparated cDNAs. Consequently, the fractionated regenerating liver cDNAs represent the entire regenerating liver cytoplasmic poly(A$^+$) RNA complexity.

Tumor cytoplasmic poly(A$^+$) RNA hybridized to regenerating liver abundant frequency cDNAs 200–10 000 times slower than the homologous RNA (Figure 3A). Furthermore, the slopes of the hybridization curves were not parallel, suggesting that the frequency distributions of the sequences represented by the abundant frequency cDNAs are very different in the two tissues. Because of the variation in hybridization rates, the possibility that the tumor reaction represented spurious hybrid formation was investigated. Table V shows that the regenerating liver abundant frequency cDNAs did not form S_1-resistant hybrids with yeast RNA, rat rRNA, or liver and tumor cytoplasmic poly(A$^-$) RNAs. A more direct analysis by comparison of the melting profiles of the hybrids was equivocal because of the inability to drive the tumor hybridization reaction to completion (Reiners, unpublished experiments). For the portion of the duplex that could be compared during the melt (70% of the hybridizable cDNA),

Table V: Hybridization Specificity of Regenerating Liver Abundant Frequency cDNAs

RNA source	species	final R_0t (M s)	double stranded (%)
regenerating liver	cytoplasmic poly(A$^+$)	10	90[a]
	cytoplasmic poly(A$^-$)	1000	5
	18 S	4000	2
	28 S	4000	3
Novikoff hepatoma	cytoplasmic poly(A$^+$)	4000	72[a]
	cytoplasmic poly(A$^-$)	4000	2
yeast	tRNA	4000	2
	whole cell	4000	3

[a] Data are from Figure 3.

the curves were similar and displaced by ~1–2 °C. These results suggest that the S_1-resistant duplexes formed between tumor RNA and regenerating liver abundant frequency cDNAs were specific.

From the hybridization kinetics and the amounts of cytoplasmic poly(A$^+$) RNA per cell, it can be estimated that there are 15 500 copies/cell of each abundant sequence in the cytoplasm of regenerating liver and 13 copies/cell in the tumor (Figure 3A, Table IV). However, because the frequency distribution of the abundant frequency RNAs is not the same in the liver and the tumor, the estimates of 13 copies/cell in the tumor may not be representative for several messenger types. Since the tumor values in Table IV were calculated by assuming an $R_0t_{1/2}$ of 65, which is 812 times slower than the observed regenerating liver $R_0t_{1/2}$, and the kinetics of hybridization with tumor RNA are 200–10 000-fold slower, the actual number of abundant frequency sequences in the tumor cytoplasm ranges from 1 to 53 copies per cell.

The hybridization kinetics of tumor cytoplasmic poly(A$^+$) RNA with less abundant frequency cDNAs are in marked contrast to the results obtained with the abundant frequency cDNAs (Figure 3B, Table IV). Tumor and regenerating liver poly(A$^+$) RNA hybridized to the less abundant frequency cDNAs with similar kinetics and to the same maximum (83–85%). These data suggest that the tumor has most of the regenerating liver less abundant frequency cytoplasmic poly(A$^+$) RNA sequences; i.e., the tumor contains the cytoplasmic sequences which constitute 99.7% of the regenerating liver cytoplasmic poly(A$^+$) RNA sequence complexity. Furthermore, the number of cytoplasmic copies per cell of these sequences is essentially the same in the two tissues.

cDNA–Nuclear RNA Hybridizations. The relative absence of regenerating liver characteristic abundant frequency RNAs in the tumor cytoplasm could be a consequence of decreased transcription of these genes or altered processing, transport, or stability of these sequences in the tumor. As an approach to this problem, nuclear RNA was isolated, separated into poly(A$^+$) and poly(A$^-$) fractions, and hybridized to the fractionated regenerating liver cDNAs. The kinetics of hybridization for the reactions of abundant frequency cDNAs with regenerating liver and tumor nuclear RNAs are shown in parts A and B of Figure 4, respectively. The cytoplasmic and nuclear hybridization curves for each tissue are parallel, suggesting that the frequency distributions of the individual RNA species, relative to one another, are similar in the cytoplasm and the nucleus. The low cytoplasmic poly(A$^+$) RNA content of abundant frequency regenerating liver RNAs in the tumor is paralleled by a low nuclear content. As in the cytoplasmic RNA comparisons, tumor nuclear poly(A$^+$) RNA hybridized 100–1000-fold slower than the homologous nuclear RNA. This is in marked contrast to the 2–10-fold difference in the rates of hybridization of tumor and liver nuclear poly(A$^+$) RNAs with the less abundant frequency cDNAs (parts A and B of Figure 5, Table VI).

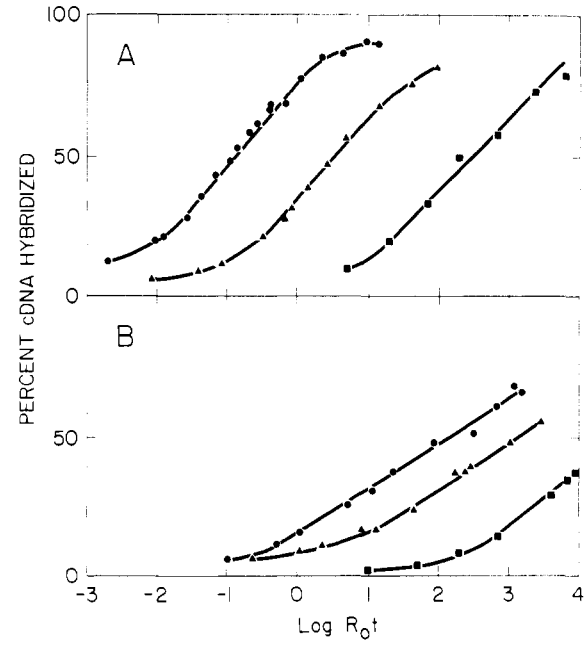

FIGURE 4: Hybridization kinetics of abundant frequency regenerating liver cDNA with regenerating liver and tumor cytoplasmic and nuclear RNAs. Regenerating liver abundant frequency cDNAs were hybridized with regenerating liver (A) or tumor (B) cytoplasmic poly(A$^+$) RNA (●), nuclear poly(A$^+$) RNA (▲), or nuclear poly(A$^-$) RNA (■). The data for cytoplasmic RNAs are from Figure 3A.

Table VI: Frequency Distribution of Abundant and Less Abundant Regenerating Liver Cytoplasmic Poly(A$^+$) RNAs in Regenerating Liver and Novikoff Hepatoma Nuclear Poly(A$^+$) and Poly(A$^-$) RNAs[a]

			nuclear RNA[b]					
			poly(A$^+$)			poly(A$^-$)		
source	frequency class	no. of RNA species	obsd $R_0t_{1/2}$ (M s)	messenger content (%)	copies/ cell	obsd $R_0t_{1/2}$ (M s)	messenger content (%)	copies/ cell
regenerating liver	abundant	39	2.4	1.23	77.0	185	0.016	11.6
	less abundant	14 500	40	27	4.6	10600	0.1	0.2
Novikoff hepatoma	abundant	39	650	0.0045	2	20000	0.00014	0.5
	less abundant	14 500	150	7.2	10	4000	0.27	1.3

[a] The table is an analysis of the data in Figures 4 and 5. [b] Hybridization curves for nuclear poly(A$^-$) reactions were carried out to R_0t values of (4–7) × 10^4 M s. $R_0t_{1/2}$ values for reactions which did not reach completion were calculated from extrapolated plots. Messenger sequence content was analyzed as described by Sippel et al. (1977) and was calculated by dividing the corrected cytoplasmic regenerating liver $R_0t_{1/2}$ (Table IV) by the observed nuclear $R_0t_{1/2}$. Copy numbers were calculated as follows: [[nuclear RNA [poly(A$^+$) or poly(A$^-$)]/ cell] × (messenger content) × (6 × 10^{23})]/(total complexity of RNA class). The total complexity estimates are given in Table IV.

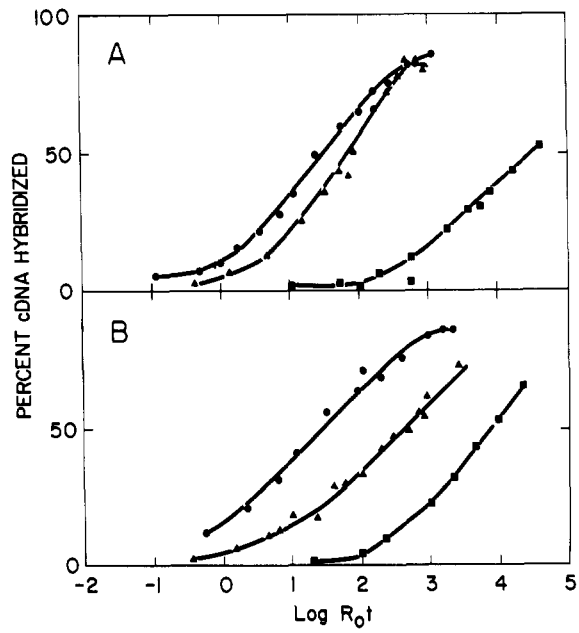

FIGURE 5: Hybridization kinetics of regenerating liver less abundant frequency cDNAs with regenerating liver and tumor cytoplasmic and nuclear RNAs. Regenerating liver less abundant frequency cDNAs were hybridized with regenerating liver (A) or tumor (B) cytoplasmic poly(A^+) RNA (●), nuclear poly(A^+) RNA (▲), or nuclear poly(A^-) RNA (■). The data for the cytoplasmic RNAs are from Figure 3B.

The regenerating liver abundant frequency RNAs also exist as nonpolyadenylated species in both the liver and tumor (parts A and B of Figure 4, Table VI). The nonpolyadenylated species constitute only 10–20% of the total nuclear content of these sequences. Furthermore, the ratios of these sequences in the nuclear poly(A^+) RNAs to nuclear poly(A^-) RNAs are similar for the two cell types. Thus, the characteristic regenerating liver abundant frequency RNAs do not specifically accumulate as poly(A^-) RNAs in the tumor.

From the estimates of cytoplasmic and nuclear copy numbers given in Tables IV and VI, it is possible to make relative comparisons of the nuclear and cytoplasmic sequence contents of specific sequences within a tissue or between tissues. Although the nuclear poly(A^+) content of regenerating liver abundant frequency RNAs in the liver is 38 times greater than in the tumor, the cytoplasmic content is 1200 times greater than that of the tumor (Tables IV and VI). Thus, there is a 32-fold accumulation of these specific sequences in the regenerating liver cytoplasm relative to the tumor. It is important to note that an analogous accumulation is also seen when the comparison is made to the less abundant frequency RNAs of the regenerating liver. The ratio of abundant to less abundant frequency regenerating liver RNAs in the liver nucleus (17) is 14 times lower than the corresponding cytoplasmic ratio (234). These data suggest that the accumulation of abundant frequency regenerating liver RNA sequences, relative to the tumor, is not due to destabilization of these sequences in the tumor but rather a preferential stabilization or increased rate of nuclear processing and transport in the regenerating liver.

The ratios of tumor cytoplasmic to nuclear poly(A^+) RNA copies per cell is identical (6.5) for RNAs defined by reaction with abundant or less abundant frequency regenerating liver cDNAs (Tables IV and VI). The analogous ratios of regenerating liver RNAs with the abundant and less abundant frequency cDNAs are 200 and 14, respectively. These data suggest that most of the regenerating liver sequences in the tumor are probably transcribed and processed and have a similar stability as the less abundant frequency RNAs in regenerating liver.

Discussion

cDNA–RNA hybridization analyses were used to compare the cytoplasmic poly(A^+) RNA populations of 18-h regenerating rat liver and Novikoff hepatoma ascites cells. As noted by others (Bishop et al., 1974; Hynes et al., 1977; Parker & Mainwaring, 1977; Meyuhas & Perry, 1979), complexity estimates are dependent upon the amount of cDNA forming hybrids, the lengths of the RNAs, and an accurate representation by the cDNAs of the diversity and relative concentrations of the template RNAs. It is difficult to ascertain that the less abundant RNAs are proportionately represented in the cDNA population, and hybridization reactions can rarely be driven to completion. Furthermore, the assumption that the average nucleotide length of total RNA is representative of RNAs in different abundancy classes must be cautiously employed in lieu of the finding that Chinese hamster ovary abundant frequency RNAs are shorter than the less abundant frequency RNAs (Meyuhas & Perry, 1979). Because of the problems associated with complexity determination, the complexity values used for calculation of sequence overlap in this study represent only relative estimates.

cDNA hybridization analyses with fractionated cDNAs suggested that the majority of the 18-h regenerating rat liver cytoplasmic poly(A^+) RNA sequence complexity was present in the cytoplasm of the Novikoff hepatoma ascites cells. The less abundant frequency regenerating liver sequences, which constituted 99% of the calculated sequence complexity, were present in similar cytoplasmic and nuclear copy numbers in both tissues and probably represent RNA species involved in cell maintenance. In addition, there may be sequences associated with growth and proliferation. The sequences of the low complexity, abundant frequency regenerating liver RNA class were 200–10 000[2] times more abundant in the cytoplasm of regenerating liver than in the tumor. The sequences constituting this RNA frequency class represented 36.5 and 0.0036–0.18% of the cytoplasmic poly(A^+) RNA masses of 18-h regenerating liver and tumor, respectively. These data suggest that phenotypic differences between cell types may be characterized by a population of RNAs which represents a very small percentage of the total sequence complexity but a large percentage of the total cytoplasmic RNA mass. Several investigators have reached similar conclusions by comparing the poly(A^+) RNAs of kidney, brain, liver, and testis (Young et al., 1976; Levy & Dixon, 1977; Hastie & Bishop, 1976) and the cytoplasmic poly(A^+) RNAs of pluripotent mouse embryonal carcinoma cells and their differentiated myogenic and erythropoietic progeny (Affara et al., 1977). Although abundant frequency sequences may be characteristic of a phenotype, they may not be responsible for regulation and maintenance of the phenotype or the differentiated state.

The low tumor cytoplasmic content of regenerating liver abundant frequency sequences was paralleled by a low nuclear content. Tumor nuclear poly(A^+) RNAs hybridized to regenerating liver abundant frequency cDNAs 100–1000-fold slower than did the homologous nuclear poly(A^+) RNAs. The differences in the kinetics of hybridization with nuclear polyadenylated RNAs were not due to altered polyadenylation

[2] The differences in the hybridization kinetics are expressed as a range because the RNA population is heterogeneous and the frequency distributions of the sequences within the population are different in the two tissues.

and accumulation of the regenerating liver abundant frequency transcripts in the tumor nuclear poly(A^-) RNA fraction. These data suggest that transcriptional modulation might be responsible for the absence of regenerating liver abundant frequency RNAs in the tumor cytoplasm.

A direct correlation between cytoplasmic and nuclear sequence abundancy has been made for several RNAs. Normal rat liver and mouse L cell cytoplasmic sequences defined as abundant by their reaction with abundant frequency cytoplasmic cDNAs were also abundant as nuclear RNAs (Sippel et al., 1977; Meyuhas & Perry, 1979). In the chick oviduct, estradiol administration results in a 1500–3000-fold frequency change in the cytoplasmic concentrations of ovalbumin and ovomucoid mRNAs (Hynes et al., 1977). The large increases in the cytoplasmic sequence contents were paralleled by an analogous increase in the nuclear concentration of these sequences (Roop et al., 1978; Tsai et al., 1978). Increased rates of preferential transcription were shown to be responsible for the increased abundancy of ovalbumin messenger (Swaneck et al., 1979).

In addition to transcriptional modulation, posttranscriptional mechanisms might also account for the differences in the cytoplasmic content of regenerating liver abundant frequency RNAs in the regenerating liver and tumor. On the basis of comparisons of the cytoplasmic and nuclear contents of the abundant and less abundant frequency RNAs, the regenerating liver abundant frequency RNAs, relative to the less abundant frequency RNAs, specifically accumulate in the regenerating liver cytoplasm. (Tables IV and VI). An analogous accumulation of the regenerating liver abundant frequency RNAs did not occur in the tumor. Rather, the tumor cytoplasmic accumulation pattern of abundant frequency liver RNAs was very similar to the accumulation patterns of regenerating liver less abundant frequency RNAs in both the tumor and liver (Tables IV and VI). These data suggest that the cytoplasmic accumulation of abundant frequency regenerating liver RNA sequences in the liver, relative to the tumor, is not due to destabilization of these sequences in the tumor but rather to a preferential stabilization or transport of these sequences in the regenerating liver. Furthermore, these data suggest that the posttranscriptional mechanisms for modulating cytoplasmic RNA abundance may be based upon the relative nuclear abundance of the RNA.

Because the kinetics of transcript appearance in the cytoplasm or messenger degradation were not measured, it is unknown whether the accumulation of regenerating liver abundant frequency RNAs is due to different rates of transcript processing and transport or preferential stabilization of the messengers. Recently, however, a direct correlation between RNA abundance and messenger stability has been reported for casein mRNA (Guyette et al., 1979), α-fetoprotein mRNA (Innis & Miller, 1979), and the cytoplasmic RNAs of mouse L cells (Meyuhas & Perry, 1979), *Drosophilia* cells (Lenk et al., 1978), and Chinese hamster ovary cells (Harpold et al., 1979).

A novel finding of this investigation is that the frequency distribution of sequences of a particular abundancy is similar in total cytoplasmic and nuclear poly(A^+) RNAs. This can be deduced from the observation that the hybridization curves for regenerating liver abundant frequency cDNAs with nuclear and cytoplasmic poly(A^+) RNAs are parallel (parts A and B of Figure 4). The hybridization curves defining reactions with less abundant frequency cDNAs are similar but not exactly parallel. However, in view of the fact that the less abundant frequency cDNAs represent a very heterogeneous RNA population and two abundancy classes (II and III, Table III), the similarity in slopes is probably significant. It is important to note that while parallel hybridization curves could be the consequence of gross nuclear RNA contamination of the cytoplasm, the amount of nuclear contamination in our experiments [0.5–4.5% of total cytoplasmic poly(A^+) RNA] could not account for the observed results.

Our hybridization results obtained with a cDNA prepared to total cytoplasmic poly(A^+) RNA are in contrast to a similar analysis performed with cDNAs synthesized from a rat liver polysomal poly(A^+) template (Sippel et al., 1977). In that investigation, curves for total nuclear RNA and polysomal poly(A^+) RNA were not parallel. However, those experiments did not take into consideration the sequences existing as cytoplasmic poly(A^+) mRNP and nuclear poly(A^-) RNA. These data suggest that nuclear poly(A^+) RNAs constituting a particular cytoplasmic abundancy class are probably processed and transported by a similar mechanism, but the frequency distribution of the sequences in the polysomes is modulated by cytoplasmic posttranscriptional regulatory mechanisms.

The identities of the abundant frequency regenerating liver cytoplasmic RNAs are unknown. However, it is reasonable that the RNAs are characteristic of the differentiated liver cell and represent liver serum proteins and enzymes involved in glycogen metabolism. Albumin mRNA, which represents 8–11% of total liver cytoplasmic poly(A^+) RNA (Tse et al., 1978), is reduced four- to fivefold in the transplantable Morris hepatoma 7777 (Tse et al., 1978; Sala-Trepat et al., 1979) and is in very low concentrations in the Novikoff hepatoma. Immunoprecipitation analyses have not detected albumin in either tumor cytoplasm or tumor RNA directed in vitro translation products.

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