Distribution and Specificity of Sequences in Polyadenylated Nuclear RNA of Normal, Regenerating, and Neoplastic Liver*

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We determined the homology between nuclear RNA of normal liver and that of regenerating and neoplastic livers. Because of the very low representation of the rare class of sequences (1 copy per cell or less) in liver nuclear RNA, very high RNA/cDNA ratios (as high as $1 \times 10^5$) were generally necessary to obtain maximal levels of hybridization. Complementary DNA prepared from 16-h regenerating liver polyadenylated nuclear RNA hybridized to the same extent with its homologous RNA and with polyadenylated nuclear RNA isolated from normal liver. We prepared, through several cycles of hybridization, a cDNA probe enriched for regenerating liver polyadenylated nuclear RNA sequences. Hybridization reactions with this enriched probe demonstrated marked differences in the abundance of classes of transcripts between nuclear RNA of normal and regenerating liver. However, the data do not support the view that there is a group of sequences which may be present in regenerating but not in normal liver. Hybridization reactions with cDNA prepared from livers with primary tumors indicated that polyadenylated nuclear RNA from those livers differs markedly from normal liver nuclear RNA in the distribution of its sequences. We conclude that the changes in the rare sequences of polyadenylated nuclear RNA during liver regeneration result from alterations in the abundance of some RNA sequences which are present in normal liver. The same conclusion applies to nuclear RNA of livers with primary tumors but in this case it was not possible to exclude the possibility that "tumor-specific" nuclear RNA sequences might exist.

Compensatory and neoplastic growth of mammalian organs have generally been conceived as processes which involve the transcription of a substantial fraction of the genome which is not active in the normal organ. Although some of the earlier work seemed to confirm these general views (1, 2), recent studies of gene expression in liver regeneration (3-5) and kidney hypertrophy (6) indicate that the mRNA populations of normal liver and kidney are qualitatively very similar to the mRNA populations present in the corresponding organ during compensatory growth.

We have shown that during liver regeneration after partial hepatectomy in rats, (a) the percentage of the genome transcribed in 12- and 24-h regenerating liver is the same as that transcribed in normal liver (3, 7); (b) there is a large increase in the amount of hepatic polysomal poly(A$^+$) mRNA starting approximately 2 h after partial hepatectomy (8); (c) polyadenylated polysomal mRNA from normal liver and that from 12-, 24-, and 72-h regenerating liver have similar sequence complexity (3, 9); and (d) shifts in the frequency distribution of polyadenylated mRNAs take place during liver regeneration (3). Our observations regarding polysomal mRNA populations during liver regeneration are in complete agreement with the work of Wilkes et al. (4) and that of Grady et al. (5). However, the published results on nuclear RNA during liver regeneration are conflicting and difficult to interpret. Wilkes et al. (4) found that the percentage of the genome transcribed in 16-h regenerating liver is the same as that of normal liver, a result which agrees with our previous observations (7). For these experiments, Wilkes et al. (4) hybridized poly(A$^+$) nRNA from normal and regenerating liver with labeled single copy rat DNA. They also demonstrated by cross hybridization experiments that adenylated nuclear RNA populations of normal and 16-h regenerating liver contain similar, if not identical sequences. However, experiments in which poly(A$^+$) nRNA was hybridized with cDNA indicated that 10-15% (by weight) of the poly(A$^+$) nRNA of 16-h regenerating liver was not present in normal liver polyadenylated nuclear RNA. Using similar techniques, Krieg et al. (10) reported that normal liver poly(A$^+$) nRNA lacked sequences present in 12-h regenerating liver poly(A$^+$) nRNA but contained all of the sequences found in polyadenylated nuclear RNA 3 h after partial hepatectomy. The results of Wilkes et al. (4) and Krieg et al. (10) are important because they may indicate the existence of "regeneration-specific" sequences which could be involved in the regulation of liver regeneration. It is, however, difficult to interpret these findings because these sequences, which appear to be of low frequency, were detected only by poly(A$^+$) RNA/cDNA hybridizations but not by hybridization of poly(A$^+$) RNA with single-copy DNA. Moreover, in contrast to these results, Grady et al. (5) reported that polyadenylated nuclear RNA from normal liver contained all of the sequences present in 2-, 5-, and 48-h regenerating liver polyadenylated nuclear RNA. They also showed that nuclear RNA from regenerating livers lacks nonadenylated RNA sequences which exist in normal liver.

Because of the conflicting results regarding nuclear RNA sequences in regenerating liver, we decided to reinvestigate the problem with the following objectives: (a) to determine if the reported differences between polyadenylated nuclear RNAs from normal and regenerating livers might represent shifts in the abundance of sequences rather than qualitative alterations in the mRNA population; (b) to construct a cDNA transcribed in normal liver. The same conclusion applies to nuclear RNA sequences found in polyadenylated nuclear RNA.

The abbreviations used are: poly(A$^+$) RNA, polyadenylated RNA; $R_{df}$, product of the initial concentration of nucleoside in moles liter multiplied by the incubation time in seconds; $E_{df}$, equivalent $R_{df}$, HAP, hydroxyapatite.

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probe which would correspond to regeneration-specific sequences in nuclear polyadenylated RNA, if these sequences do exist; (c) to determine if polyadenylated nuclear RNA from normal and regenerating livers hybrize to the same extent with repeated DNA sequences. In addition to these studies, we report on the frequency distribution of sequences of polyadenylated nuclear RNA isolated from livers which contain primary tumors.

**MATERIALS AND METHODS**

**Animals and Surgical Procedures**—The animals used for experiments with regenerating liver were 140-160-g male rats, Sprague Dawley-Holtzman, purchased from Charles River Laboratories (strain Crl:CD(SD)BR). These animals were kept in temperature-controlled rooms with alternating 12-h dark-light cycles. Partial hepatectomies were done using the technique of Higgins and Anderson (11) with continuous oxygen-ether anesthesia as previously described (12). Rats were not fed after the operation and were killed 16 h after partial hepatectomy (between 9-11 a.m.). Control animals for these experiments were sham-operated, that is, their livers were manipulated but not removed. Sham-operated rats were killed 16 h after the procedure and RNA obtained from these animals is referred to in the text as “normal liver” RNA.

Rats used in the carcinogenesis experiments were Sprague-Dawley rats purchased from Charles River Laboratories (strain Crl:CD(SD)BR). Animals weighing 140-160 g were fed a choline-deficient diet containing 0.05% ethionine (Teklad Test Diets) according to the procedures of Shinozuka et al. (13) and as described in our previous work from our laboratory (14). These animals were killed 32 weeks after the start of the feeding. Livers which showed extensive changes were used for RNA extraction. Histological analysis of these livers revealed the presence of areas of well differentiated hepatocellular carcinoma, preneoplastic nodules, and a variable degree of cholangiofibrosis (14, 15). Control animals for these experiments were fed a normal diet.

**Isolation of Nuclear RNA**—The method used for isolation of nuclei is based on that described by Buech et al. (16) with some modifications (6). Livers were removed from the animals immediately after killing, cut into small pieces, rinsed, and homogenized in 5 volumes of 2% citric acid in a Potter-Elvehjem homogenizer. The homogenates were centrifuged at 600 X g for 10 min at 2°C. The pellets were resuspended with a solution containing 250 mM sucrose, 50 mM Tris (hydroxymethyl)aminomethane, pH 7.4, 25 mM NaCl, 5 mM MgCl2 (TMN buffer), containing 250 mM sucrose, 50 mM Tris (hydroxymethyl)aminomethane, pH 7.4, and incubated for 15 min at 4°C with iodosaccharate-treated deoxyribonuclease. Benzonase was added (2% final concentration) and the suspension shaken for an additional 15 min. One-eighth volume of 10 X SET buffer (100 mM sodium lauryl sulfate, 50 mM EDTA, 250 mM Tris, pH 7.4) was added followed by phenol/chloroform (1:1) saturated with SET buffer at a volume equal to that of the suspension. The extraction was carried out for 15 min at room temperature. After centrifugation, the aqueous phase was saved and the interphase and organic phase were extracted with 1 X SET buffer at 65°C for 5 min. The aqueous phases of the extraction were combined and extracted four more times with phenol/chloroform/isoamyl alcohol (v:v:v=25:25:1), and the final mixture was passed through a mixture of isoamyl alcohol and chloroform (1:24). The RNA was precipitated overnight at -20°C in 2 volumes of 95% ethanol containing 1 M NaCl. The RNA was digested again with deoxyribonuclease, extracted with phenol, repurified, and after resuspension, passed through a Chelex 100 (Bio-Rad Laboratories) column, repurified and stored at -70°C. The characteristics of the nuclear RNA preparations obtained from normal, regenerating, and neoplastic liver were similar to those previously described (8).

**Preparation of Poly(A)+RNA**—Polyadenylated nuclear RNA was isolated from liver nuclei by affinity chromatography on poly(U)-Sepharose columns as previously described (3, 9). For a few experiments, only material passed only through Sephadex G-50 and cDNA preparations passed through Sephadex and then fractionated by centrifugation gave identical results in hybridization with RNA. Polyadenylated RNA Hybridization—Approximate amounts of polyadenylated RNA at concentrations varying from 50 to 7000 μg/ml were mixed with [3H]cDNA at RNA/DNA ratios of 8 X 10 to 1 X 105. The nick-translated DNA was mixed, lyophilized to dryness, and dissolved in a solution containing 0.24 or 0.4 M sodium phosphate buffer (pH 6.9), 1 mM EDTA, and 0.05% sodium dodecyl sulfate (3, 9, 14). The hybridization mixtures were incubated in sealed, sterile, siliconized capillary tubes at 68°C, after boiling for 5 min to denature the nucleic acids. All reactions were terminated by immersing the capillary tubes in ethanol/2 M NaCl. Conditions for the S1 nuclease digestion have already been described (6, 14). Zero time values (1-4%) have been subtracted from all hybridization data. The hybridization curves were fitted and analyzed using the computer program described by Pearson et al. (17) and modified by us (18) to include the calculations of derivatives as described by Quinlan et al. (19).

**Preparation of Repetitive DNA**—Repetitive DNA was prepared from sheared rat DNA using a method based on that of Britten et al. (20). Rat DNA was sheared using a Virtis 60 K homogenizer (9) and the repetitive fraction was isolated after annealing of the material in 300 mM NaCl, 100 mM NaPO4, 1.4% piperazinediethanesulfonic acid, pH 7.4, to a Ca of 40. The hybridized material contained duplicates of repetitive sequences (averages of 300 base pairs) with interspersed single-stranded DNA. To remove the single-stranded DNA, the material was digested with S1 nuclease extracted twice with chloroform/isoamyl alcohol, and passed through a Sephadex-G-100 column. Two micrograms of repetitive DNA were labeled by nick translation for 12 h at 12°C in an incubation mixture containing DNA polymerase I (Boehringer Mannheim Biochemicals) but no nuclease (21). “Snapback” DNA was eliminated by passing the material through a HAP column (21). The single-stranded DNA was dialyzed, passed through a Chelex-100 column and stored at -70°C. The isolated DNA annealed with sheared total rat DNA with the characteristics expected for repetitive DNA (9).

**RESULTS**

**Complexity and Abundance of Nuclear and Polysomal Polyadenylated RNA in Normal Rat Liver**—To determine the complexity and sequence abundance of polyadenylated nuclear RNA, we isolated RNA by Poly(U)-Sepharose chromatography as previously described (3, 9, 14) or by three passages of the material through oligo(dT)-cellulose columns. Hybridization of these RNAs with their respective cDNAs generated identical curves regardless of the isolation method used. Fig. 1 presents a derivative plot of the hybridization of nuclear RNA with its cDNA and, for comparison, the derivative of the hybridization of polyadenylated polysomal mRNA with its cDNA. For the analysis of these curves (Table I), we assumed that the sequences in the RNA populations are separated into three frequency classes. Each component of the nuclear RNA hybridization curve has a Rmax value which is 15-70-fold higher than that of cytoplasmic RNA and the difference is more marked for the first component of the curves. The slower hybridization kinetics of nuclear RNA is due in part to the larger size of nuclear RNA (8) but it primarily reflects the higher complexity of the RNA and the lower frequency of its sequences. In contrast to polysomal RNA which contains more than 5000 copies/cell of some sequences, the most abundant sequences in nuclear RNA are
represented by approximately 30 copies each. The least abundant sequences are present in polysomal RNA at about 5 copies/cell and at 1 copy for each 2–4 cells in nuclear RNA. Obviously, these analyses are only approximations and assume that RNA sequences are evenly distributed through the various cell populations in the liver.

Hybridization of Nuclear RNA from Normal and Regenerating Liver with cDNA—cDNA synthesized from 16-h regenerating liver polyadenylated nuclear RNA was hybridized with its homologous RNA and with polyadenylated nuclear RNA from normal liver (heterologous hybridization). The hybridizations were carried to E, Rds of approximately 5 x 104, using an RNA excess of approximately 106 for the higher Rd points. The curves for the homologous and heterologous hybridization, shown in Fig. 2, have almost identical kinetics. The heterologous hybridization values are slightly lower between E, Rds of 103 and 104 but the saturation levels are the same for both curves. This indicates that polyadenylated nuclear RNA from 16-h regenerating liver does not appear to contain “regeneration-specific” sequences which are absent from normal liver. The same experiments were repeated using normal and regenerating liver RNA isolated by three passages through oligo(dT)-cellulose columns. The cDNA prepared from 16-h regenerating liver nuclear RNA was centrifuged in alkaline sucrose gradients and the material larger than 6 S was saved. The curves obtained from the hybridization of this cDNA with normal or regenerating liver nuclear RNA (isolated by oligo(dT)-cellulose chromatography) were identical to those shown in Fig. 2 (data not presented).

**Construction of a cDNA Probe Containing Regeneration-specific Sequences**—To search more directly for sequences present in regenerating but not in normal liver, we attempted to construct a cDNA probe which would be enriched for these sequences if they indeed exist. We followed the protocol shown in Table II to construct such a probe. Polyadenylated nuclear RNA from normal liver was hybridized with cDNA prepared from 16-h regenerating liver RNA to a E, Rds of 4 x 106 under

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**TABLE I**

<table>
<thead>
<tr>
<th>Classes</th>
<th>cDNA hybridized</th>
<th>Rds (obs)</th>
<th>Rds (corr)</th>
<th>Complexity</th>
<th>Copies/cell</th>
</tr>
</thead>
</table>
| Nuclear Poly (A') RNA  
I   | 26.8 | 3.24 | 1.0 | 555 | 28 |
| II  | 38.1 | 53 | 23.2 | 12,894 | 2 |
| III | 22.0 | 630 | 159 | 88,333 | <1 |
| Polysomal Poly(A') RNA  
I   | 23.4 | 0.044 | 0.0125 | 20 | 5,219 |
| II  | 35.8 | 1.005 | 0.433 | 698 | 160 |
| III | 23.9 | 45.9 | 13.30 | 21,290 | 4 |

* Corrected \( R_{td} = \frac{R_{td}}{R_{cell}} \) if that class were present alone in RNA population.

* Number of different sequences of average nucleotide length present in nuclear or polysomal poly(A') RNA.

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**TABLE II**

**Construction of cDNA probe to detect regeneration-specific sequences**

- cDNA from poly (A') regenerating liver nuclear RNA × poly (A') nuclear RNA from normal liver
  - HAP chromatography
    - hybridized cDNA (75%) × nonhybridized cDNA (25%)
      - HAP chromatography
        - nonhybridized cDNA (19.25%) × hybridized cDNA (5.7%)
          - HAP chromatography
            - hybridized cDNA (5.14%) × nonhybridized cDNA (0.56%)
              - (Probe)

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* Pol (A+) RNA/ cell × fraction of cDNA hybridized × 6 × 10^12 base sequence complexity of RNA class × RNA size
the conditions described in the legend of Fig. 2 using an RNA excess of $2 \times 10^3$. At the end of the reaction, the hybridization mixture was chromatographed on a HAP column. The non-hybridized fraction was eluted with 0.12 M phosphate, precipitated with alcohol, treated with 0.5 N NaOH and passed through a Sephadex G-50 Chelex column. The recovered cDNA, which constituted 25% of the original material and was 95% precipitable with trichloroacetic acid, was hybridized with polyadenylated nuclear RNA from 16-h regenerating liver. After the reaction, the hybrids were isolated by elution from a HAP column with 0.4 M phosphate and were treated as described above for the first round of hybridization. Approximately 80% of the cDNA remained single-stranded in the homologous hybridization, indicating that most of the material which did not anneal in the heterologous hybridization was not capable of annealing with homologous RNA. The cDNA which annealed in the second round of hybridization (5.7% of the original cDNA) was again hybridized with nuclear RNA from normal liver. The cDNA which remained single-stranded in this round of hybridization corresponded to 0.56% of the starting cDNA and was used as a probe (referred to as "e cDNA") in subsequent experiments. Fig. 3 shows the hybridization of the e cDNA probe with poly(A') nRNA from normal and 16-h regenerating liver. For the high E Rot points, the RNA excess was approximately $1 \times 10^3$. The heterologous hybridization is slower which indicates that there are differences in the abundance of certain sequences between poly(A') nRNA of normal and regenerating liver. However, e cDNA hybridizes to the same extent with normal and regenerating liver poly(A') nRNA. Thus, the curves do not demonstrate the existence of "regeneration-specific" sequences in poly(A') nRNA from 16-h regenerating liver.

Apparent Saturation Levels of Heterologous Hybridization Curves—Liver cells contain, at most, 1 copy/cell of the sequences which comprise the most complex class of polyadenylated nuclear RNA. Alterations in the abundance of these sequences in regenerating liver poly(A') nRNA might falsely appear as a qualitative change relative to normal liver poly(A') nRNA if the RNA in the hybridization mixture is not in sufficient excess of the cDNA. This problem is illustrated in Fig. 4. Fig. 4a shows the heterologous hybridization of the e cDNA probe (used in Fig. 3) with normal nuclear RNA. For this reaction, we used three different RNA concentrations in the hybridization mixture: 0.4, 2.5, and 6 mg/ml. At RNA concentrations of 2.5 mg/ml, the reaction levels fell off when approximately 66% of the cDNA has been hybridized. However, at this RNA concentration, the rare RNA sequences are probably not in sufficient excess to carry the reaction further because, at approximately the same E Rot values, an
additional 12% of the cDNA is hybridized when the RNA concentration is increased to 6 mg/ml. These results show that the real saturation level for this reaction is reached only when there is an approximately $6 \times 10^6$ or higher excess of RNA over DNA in the hybridization mixture. Fig. 4b shows the hybridization of cDNA synthesized from 16-h regenerating liver with normal and regenerating liver polyadenylated nuclear RNA. These results are the same as those shown in Fig. 2 but the RNA/cDNA ratio used for the higher Rot points was decreased from $1 \times 10^6$ to $2 \times 10^6$. Despite the scatter of the points in the homologous reaction, it is apparent that the two curves shown in Fig. 4 have different saturation levels in contrast to the curves in Fig. 2. The saturation value (computer fit) of the homologous curve is approximately 8% higher (range 4-12%) than that of the heterologous hybridization. The difference between the saturation levels of the two curves could be misinterpreted as indicating the existence of a substantial fraction of regeneration-specific sequences. However, the results obtained actually reflect differences in the frequency of rare RNA sequences between normal and regenerating liver and the lack of a sufficient excess of normal liver RNA to anneal with the rare sequences represented in the cDNA.

**Hybridization of Nuclear RNA with Repetitive DNA**—The data presented in this paper indicate that normal and regenerating liver polyadenylated nuclear RNA might differ only in the abundance but not in the kinds of sequences they contain. However, the observations of Wilkes et al. (4) are compatible with the idea that "regeneration-specific" sequences could be transcribed from repetitive DNA. To examine this possibility, we hybridized unfractionated nuclear RNA (containing poly(A+) and poly(A-) molecules) from normal and 16-h regenerating liver with $^3$H-repetitive DNA. Two conditions were used for these annealing reactions, shown in Fig. 5. Under less stringent conditions (0.41 M phosphate buffer at 55 °C, Fig. 5a), the saturation level of the normal liver RNA reaction with repetitive DNA is approximately 11% higher than that of the regenerating liver RNA curve. Under more stringent conditions (0.24 M phosphate buffer at 68 °C, Fig. 5b) the saturation levels of the curves are the same. These results do not support the hypothesis that some sequences coded from repetitive DNA might be regeneration-specific.

**Homologous and Heterologous Hybridizations with cDNA**

- **Synthesized from RNA of Livers with Primary Tumors**—We isolated polyadenylated nuclear RNA from livers of rats kept on a choline-deficient diet containing 0.05% ethionine. These livers had multiple carcinomas, of average diameter of 0.5-1.0 cm, in addition to areas of hyperplasia and preneoplastic nodules (14, 15). The cDNA prepared from these livers was hybridized with its homologous RNA and with polyadenylated nuclear RNA from normal liver (Fig. 6). The heterologous hybridization is considerably slower than the homologous one, indicating that the abundance of some nuclear RNA sequences present in the tumor is considerably lower in normal liver. Moreover, since there is a small difference in the saturation level of the two curves, it is possible that some of these sequences are absent from normal liver and could be considered as being specific to livers with neoplasia but this conclusion cannot be firmly made with this type of experiment (see "Discussion").

The changes in RNA frequency distribution can be better appreciated by a derivative plot of the curves from Fig. 6. For these plots (Fig. 7) the curves were arbitrarily analyzed as if

**Fig. 5.** Hybridization of nRNA from normal and regenerating liver with repetitive liver (see "Materials and Methods") was hybridized with nuclear RNA (unfractionated) of normal (○) and 16-h regenerating liver (□). a, reaction at 55 °C in 0.41 M phosphate buffer (pH 6.8), 1 mM EDTA, 0.05% sodium dodecyl sulfate; and b, reaction at 68 °C in the same mixture but containing 0.24 M phosphate buffer. RNA/DNA ratios used in these reactions were $6.7 \times 10^6$ to $1.3 \times 10^6$. The extent of hybridization was determined by S1 nuclease digestion. The data presented have been corrected for DNA-DNA hybridization by treating the hybridization mixture with ribonucleases A and T1 (3, 7).

**Fig. 6.** Homologous and heterologous hybridization of cDNA synthesized from poly(A+) nRNA from neoplastic livers. Complementary DNA was prepared from poly(A+) nRNA isolated from livers of rats fed a choline-deficient diet containing 0.05% ethionine for 32 weeks. The conditions of hybridization are the same as those indicated in the legends to Figs. 1 and 2. Hybridization of cDNA prepared from neoplastic liver with homologous poly(A+) nRNA (Δ—Δ) or poly(A+) nRNA from normal liver (○—○).

**Fig. 7.** Derivative plots of the hybridization of cDNA prepared from neoplastic liver with homologous and heterologous RNA. The curves shown in Fig. 6 were plotted according to the method described by Quinlan et al. (19). Hybridization of cDNA prepared from poly(A+) nRNA of neoplastic livers with homologous RNA (●—●) or poly(A+) nRNA from normal liver (■—■).
they contained three abundance classes. The homologous curve (dashed line) has components with \( E_{\text{RDA}} \) of approximately 5, 100, and 700 which comprise 23, 28, and 49% of the hybridized cDNA, respectively. The hybridization of the same cDNA with normal liver nuclear RNA (solid line) shows a different distribution of sequences and the whole curve is displaced to the right, indicating the lower frequency of some RNA sequences in normal liver. The three components of the heterologous curve represent 44, 95, and 5% of the hybridized cDNA, and have an opposite distribution of frequencies than that found in the homologous reaction. The data suggest that sequences which are abundant in normal liver nuclear RNA became less frequent in the liver with neoplasia and that a reverse phenomenon takes place for some rare sequences of normal liver RNA.

**DISCUSSION**

Analyses of changes in the rare class of nRNA sequences during liver growth are difficult because of the low frequency of these molecules, a factor which probably accounts for the conflicting results published in the literature. The distribution of sequences in rat liver poly(A\(^+\)) nRNA is much narrower than that in polysomal poly(A\(^+\)) RNA. Liver nuclei each contain no more than 50–100 copies of the abundant sequences and at the most, 1 copy of the rare sequences. These rare sequences more likely occur at 1 copy/2–4 cells. These estimates for the rare sequences agree with the results of Grady et al. (22), who based their calculations on the hybridization of liver nuclear RNA with a unique DNA probe representing expressed genes. Sippel et al. (23) and Chikarashii et al. (24) estimated the abundance of rare sequences of liver nuclear RNA as 5 copies/cell and 1 copy/4 cells, respectively. The narrow distribution of frequencies in nuclear RNA and the very low representation of rare sequences are probably a general feature of mammalian cells given that the results of Baimain et al. (25) with Friend cells are very similar to those reported here.

A logical interpretation of the results presented in this paper is that the changes in the rare sequences of polyadenylated nuclear RNA during liver regeneration result from alterations in the abundance of some RNA sequences which are present in normal liver. In the first stage of our experiments, we hybridized cDNA synthesized from 16-h regenerating liver poly(A\(^+\)) nRNA with its homologous RNA and with polyadenylated nRNA isolated from normal liver and found no qualitative differences between these two RNA populations. However, this type of analysis might not detect substantial differences which may exist between RNA populations, as illustrated by the work of Grouse et al. (26). They showed that qualitative differences between the RNA populations of differentiated and undifferentiated neuroblastoma cells were not apparent when nonselected cDNA was used in hybridization reactions but could be demonstrated with constructed cDNA probes enriched for sequences of either RNA population. We followed a similar protocol and prepared, through several cycles of hybridization, a cDNA probe enriched for regenerating liver poly(A\(^+\)) nRNA sequences. Hybridization reactions with this probe demonstrated marked differences in the abundance of classes of transcripts between nuclear RNA of normal and regenerating liver. However, the data do not support the view that there is a group of nRNA sequences which are present in regenerating but not in normal liver. Because the methods used in this work were designed for the analysis of complex RNA populations, one cannot entirely exclude the possibility that there are regeneration-specific sequences in 16-h regenerating liver nRNA. However, much more sensitive methods involving the cloning of individual sequences will have to be used to prove the existence of specific sequences. Wilkes and Birnie (27) have recently attempted to isolate regeneration-specific sequences from nuclear RNA using mercerated RNA and thioc-SePharose chromatography. These procedures led to a relatively small enrichment of the original cDNA yielding a probe which reacted extensively with polyadenylated nuclear RNA from normal liver.

It is essential to use a very large excess of RNA in heterologous hybridization reactions involving nRNA to obtain the highest attainable levels of annealing. We obtained maximal hybridization between normal liver poly(A\(^+\)) nRNA and cDNA prepared from regenerating liver RNA with RNA/cDNA ratios of approximately 1 \( \times 10^5 \). At an RNA/cDNA ratio of 2 \( \times 10^5 \), which is often used in this kind of experiment, the saturation level of the reaction was 9–12% below the maximum value. These findings might explain the discrepancies between our results and those of others (4, 10) and provide an explanation for some of our earlier data regarding qualitative differences in polysomal mRNA of regenerating liver which were not confirmed by our own subsequent work or by others (3–5).

If polyadenylated nuclear RNA of normal and regenerating liver differ from each other only in the frequencies in which various sequences are represented, it is expected that these two RNA populations would hybridize to the same extent with unique DNA. This is indeed the case, as shown in our previous work (3, 7) and that of Wilkes et al. (4) and Grady et al. (5, 22). In addition, we show in this paper that nuclear RNA from normal and regenerating liver anneal to the same extent with repetitive rat DNA.

The results presented in this paper show that polyadenylated nuclear RNA from livers with primary hepatocellular carcinomas differs markedly from normal liver nuclear RNA in the distribution of its sequences. The abundant class of transcripts from neoplastic liver hybridizes with 23% of the homologous cDNA. In contrast, the most abundant class of sequences in the normal liver nuclear RNA corresponds to 44% of this cDNA. We had previously shown with the same carcinogenesis system that liver polysomal mRNA of rats fed the carcinogenic diet contained at a much reduced frequency transcripts which are abundant in normal liver. At the same time, we found no indication for the existence of "tumor-specific" sequences in polysomal mRNA (14). Our subsequent work with a transplantable hepatoma showed that tumor polysomal mRNA lacks 10–15% (by mass) of normal liver mRNA sequences and does not contain groups of sequences that could be considered to be tumor-specific (18). These findings regarding polysomal polyadenylated RNA system are similar to those reported for a hepatoma cell line and for primary and Novikoff hepatomas (28–31). However, there is less agreement between studies of nuclear RNA changes in primary and transplantable hepatomas reported by various laboratories. Jacobs and Birnie (28) found little difference between the sequences of nuclear RNA of normal liver and those of an hepatoma cell line while other reports indicate that nuclear RNA from transplanted hepatomas has lost a substantial proportion of sequences which occur in normal liver nuclear RNA (31, 32).

It is possible that livers with primary tumors contain nuclear RNA sequences not present in normal liver. However, the small magnitude of the differences found and the marked cellular heterogeneity of livers of animals fed the carcinogenic agent lead us to conclude that this issue cannot be settled with the type of approach used in the present studies. The problem might be investigated more directly by examining the expression of individual sequences in the nuclear RNA of
the various cell types found in neoplastic and preneoplastic livers.

Despite the uncertainties, it is fair to conclude that alterations in the abundance of transcripts, rather than qualitative differences, constitute the more obvious changes detected in nuclear and cytoplasmic polyadenylated RNA during liver regeneration. In preneoplastic and primary tumors, these alterations are more marked and, in transplantable hepatomas, are accompanied by evidence of genetic restriction. So far, studies with complex liver RNA populations have not unequivocally demonstrated sequences which are specific for liver regeneration or neoplasia or are secondarily involved in these processes already exist in the normal liver.

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