Tumor-specific Polyadenylated RNA's from 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumors Revealed through Hybridization with Fractionated Single-Copy DNA

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ABSTRACT

Polyadenylated (poly(A)+) and nonpolyadenylated (poly(A)−) RNA fractions from primary, hormone-dependent, 7,12-dimethylbenz(a)anthracene-induced mammary tumors and normal rat mammary tissue were analyzed by molecular hybridization to determine if there is a unique class of "tissue-specific" RNA sequences. Single-copy [3H]DNA was fractionated into probes which were either depleted of midpregnant RNA sequences or complementary to these same sequences. When these probes were annealed to homologous and heterologous poly(A)+ RNA's, distinct sets of single-copy sequences were found in the two poly(A)+ RNA populations. The complexity of the "tumor-specific" sequences was about 6.7 x 10^7 nucleotides (assuming asymmetrical transcription).

Hybridization of the poly(A)− RNA fractions isolated from the 7,12-dimethylbenz(a)anthracene-induced tumors and the normal mammary gland to poly(A)+ complementary DNA revealed that both tissues appear to contain a class of poly(A)+ RNA sequences which were distinct from the set of poly(A)+ RNA's. Both poly(A)− RNA fractions saturated at 3% when hybridized to single-copy [3H]DNA. However, approximately 15 to 25% (minimum estimate) of the complexity of the poly(A)− RNA was contributed by contaminating poly(A)+ RNA sequences. Therefore, the actual complexity of the poly(A)− RNA fractions was 2.0 to 2.5% of the single-copy hybrid genome. Mixtures of these two RNA fractions also gave a saturation value of 3.0% when annealed to single-copy [3H]DNA. This indicated that most of the poly(A)− RNA's were similar in the neoplastic and normal mammary tissues. Although the majority of the infrequent RNA species are held in common between the two tissues, there is a distinct set of high-complexity poly(A)+ RNA sequences, presumably of nuclear origin, which is unique to the 7,12-dimethylbenz(a)anthracene-induced tumors. Whether these RNA's are processed and became functional messenger RNA's is not known. Presumably, they play a role in regulating the frequencies of the tissue-specific abundant and moderately abundant messenger RNA's and therefore provide one mechanism by which the concentration of specific proteins and ultimately the expression of the transformed phenotype may be regulated.

INTRODUCTION

Mammary tumors induced in rats by the carcinogen DMBA have been widely studied as a model system for hormone-dependent mammary cancer (19). While these tumors retain hormonal dependence for growth, they exhibit a number of changes from the normal phenotype, i.e., loss of differentiated function and morphological changes (1, 32, 35), which may be the result of specific alterations in their pattern of gene expression. Previously in our laboratory, the techniques of molecular hybridization and cell-free translation were utilized to analyze total cellular poly(A)+ RNA populations from hormone-dependent, DMBA-induced mammary adenocarcinomas and normal 14-day midpregnant glands in Sprague-Dawley rats (36). These studies were designed to elucidate any marked changes in gene expression which might occur in these primary hormone-dependent mammary tumors, resulting in a deviation from normal hormone-regulated growth and differentiation.

Homologous and heterologous hybridizations to cDNA of poly(A)+ RNA populations from the neoplastic and normal tissues indicated that the mRNA's which code for maintenance functions are probably shared between the tumors and the normal mammary tissue. Changes in the frequencies of the tissue-specific abundant and moderately abundant mRNAs may provide one mechanism by which the concentration of specific proteins and ultimately the expression of the transformed phenotype may be regulated. Hybridization of both poly(A)+ RNA populations to single-copy [3H]DNA showed that the majority of the infrequent RNA's species also appear to be held in common between the 2 tissues. These studies, however, did not preclude the existence of a set of tumor-specific sequences required for maintenance of the transformed phenotype.

Saturation hybridization with single-copy DNA does not afford the discrimination to detect subtle differences in the scarce or complex RNA abundance class between 2 RNA populations (7). A more accurate method of detecting qualitative differences between 2 total RNA populations involves isolating single-copy DNA from DNA-RNA hybrids and then reacting this probe with homologous and heterologous RNA's (7, 10, 11). In this study, single-copy [3H]DNA was, therefore, fractionated into midpregnant mDNA and null mDNA. By performing hybridization reactions with these fractionated probes to poly(A)+ RNA's from the DMBA-induced mammary tumor and the normal tissue, we were able to ask the following question. Is there a DNA complementary to polyadenylated RNA isolated from mammary glands from 14-day pregnant animals; null mDNA, unique sequence DNA devoid of sequences complementary to polyadenylated RNA isolated from mammary glands from 14-day pregnant animals; poly(A)+, nonpolyadenylated; oligo(dT), oligodeoxythymidyl acid; poly(A), polyadenylid acid; poly(U), polyuridylic acid; HAP, hydroxyapatite; Rg, RNA concentration (mol/liter) x time (sec); PB, phosphate buffer (equal molar amounts of disodium and monosodium); Cc, DNA concentration (mol/liter) x time (sec); Cc1/2, the Cc value where 50% of the sequence component has reassociated; kb, kilobase(s).
distinct set of "tumor-specific" RNA sequences which we were previously unable to detect by either cDNA complexity analysis or saturation hybridization with unfraccionated single-copy DNA?

In addition to our studies on poly(A)+ RNA's from DMBA-induced tumors and normal mammary tissue, we examined the poly(A)− RNA fraction from these tissues to determine if we could detect unique sets of poly(A)− RNA's. Earlier studies on poly(A)− RNA's indicated that most of the RNA complexity was represented in the poly(A)+ RNA fraction and that the majority of nonribosomal poly(A)− sequences were a subset of the poly(A)+ fraction (2, 18). Recent studies, however, have demonstrated the presence of a high-complexity unique class of poly(A)− RNA's in mouse brain (38) and rat brain (5).

In light of these recent findings, we asked the following questions. (a) Is there a distinct class of poly(A)− RNA present in the tumor and the normal mammary glands? (b) If such an RNA population exists, are there significant qualitative differences in this RNA class between the 2 tissues which can be detected by saturation hybridization with single-copy DNA? To address these questions, poly(A)+ RNA fractions were, therefore, utilized as templates for cDNA synthesis for subsequent hybridization reactions with poly(A)+ and poly(A)− RNA's from the neoplastic and normal tissues to determine if the majority of the poly(A)− RNA's were simply a subset of poly(A)+ RNA species.

In this paper, we report that the use of "recycled" single-copy DNA probes greatly increases the sensitivity of the saturation hybridization assay. While the majority of the complex scarce RNA sequences are held in common between the neoplastic and normal tissue, we were able to detect a distinct set of high-complexity tumor-specific RNA sequences. Furthermore, both the DMBA-induced tumors and normal mammary tissue appeared to contain a unique poly(A)− RNA population, although both poly(A)− RNA classes had a significant subset of RNA species derived from the poly(A)+ RNA class. As had been shown previously for poly(A)+ RNA, the majority of poly(A)− RNA's were shared between these 2 tissues.

**MATERIALS AND METHODS**

**Tumor Induction.** Primary mammary carcinomas were induced in 50-day-old female Sprague-Dawley rats by DMBA as described previously (36). To optimize reproducibility in the RNA preparations from these tumors, the following procedures were followed. Most early appearing DMBA-induced mammary tumors are hormone dependent (4). Therefore, only rapidly growing, small tumors, usually 1 to 2 sq cm, that appeared during this time were utilized in these experiments to minimize necrosis. To ensure that these tumors were adenocarcinomas, histological examinations were performed on the tumors. Animals were sacrificed by cervical dislocation, and the dissected tumors were rinsed in cold 0.9% NaCl solution and quick frozen in liquid N2. Normal mammary tissues were obtained from 14-day midpregnant Mammary glands were removed and quick frozen, as described previously (33).

**Isolation of Poly(A)+ and Poly(A)− RNA's.** Total nucleic acids were extracted from quick-frozen DMBA mammary tumors and 14-day mid-pregnant mammary glands as described previously (24, 31, 36). Fractionation of total RNA into poly(A)+ and poly(A)− RNA fractions was accomplished by 3 passages over oligo(dT)-cellulose as described in detail elsewhere (31, 36). To remove contaminating DNA sequences from the poly(A)− and poly(A)+ RNA samples, they were treated with afffinity column-purified DNAse I as described by Nordstrom et al. (28). The fraction which bound to oligo(dT)-cellulose in high salt was designated as poly(A)+ RNA, while the unbound fraction was designated as poly(A)− RNA.

The amount of poly(A) present in the poly(A)+ and poly(A)− RNA's was estimated by hybridization with [3H]poly(U). The [3H]poly(U) was hybridized to poly(A) standards and various dilutions of poly(A)+ and poly(A)− RNA. After digestions with S1 nuclease, the amount of poly(A) hybridized to [3H]poly(U) was quantitated, as described previously (17).

**Synthesis of cDNA Probes.** Representative [3H]DNA probes to poly(A)− RNA's from the tumors and normal mammary gland were synthesized in vitro using calf thymus DNA fragments as random primers. The experimental conditions are described in detail elsewhere (30). The specific activity of these probes was 5.0 × 10^6 cpn/μg DNA's prepared from poly(A)+ RNA contained sequences complementary to RNA. These sequences were removed by HAP chromatography following hybridization to a 100-fold excess of chicken rRNA. Approximately 75% of the mass of the cDNA was removed following 2 cycles of HAP chromatography.

**Preparation of Midpregnant mDNA and Null mDNA.** Midpregnant mDNA and null mDNA was isolated from single-copy [3H]HindIII DNA by a modification of the method of Kamalay and Goldberg (16). Single-copy [3H]DNA was hybridized with a 1200-fold mass excess of poly(A)+ RNA isolated from 14-day midpregnant mammary gland. This RNA preparation was treated previously with DNase to remove contaminating DNA sequences. Following hybridization to a RH of 50,000, the reaction mixture was digested with RNase A (5 μg/ml) for 1 hr in a high-salt solution (0.25 M PB) at room temperature to remove unhybridized RNA. RNase was removed by digestion with protease K (300 μg/ml) and extraction with phenol-chloroform-sodium dodecyl sulfate. The mixture was then passed over HAP at 60°C in 0.14 M PB containing 0.1% sodium dodecyl sulfate. The single-copy [3H]DNA which did not bind to HAP was used to prepare null mDNA while the [3H]DNA in DNA-RNA hybrids was used to prepare mDNA.

The bound single-copy [3H]DNA which contained midpregnant RNA-DNA duplexes as well as DNA-DNA duplexes was treated with RNase A (10 μg/ml in 0.05 M PB at room temperature) and protease K and extracted with phenol-chloroform-sodium dodecyl sulfate as described above. Following rechromatography on HAP, the [3H]DNA which was not hybridized was treated with 0.2 N NaOH, neutralized, and precipitated in ethanol. This fraction was designated null mDNA, and the yield was 20% of the theoretical maximum.

The bound single-copy [3H]DNA which contained midpregnant RNA-DNA duplexes as well as DNA-DNA duplexes was treated with RNase A (10 μg/ml in 0.05 M PB at room temperature) and protease K and extracted with phenol-chloroform-sodium dodecyl sulfate as described above. This step was designed to destroy the RNA in the DNA-RNA hybrids. This mixture was chromatographed on HAP in 0.14 M PB. The single-stranded [3H]DNA which was in RNA-DNA hybrids was collected, while the small percentage of [3H]DNA-RNA hybrids was discarded. This fraction designated as midpregnant mDNA was dialyzed, concentrated, and rehybridized to a 500-fold excess of midpregnant poly(A)+ RNA to a RH of 25,000. The reaction mixture was then treated with RNase and proteinase K and extracted with phenol-chloroform-sodium dodecyl sulfate as described above. Following rechromatography on HAP, the [3H]DNA which was not hybridized was treated with 0.2 N NaOH, neutralized, and precipitated in ethanol. This fraction was designated mid mDNA, and the yield was 25% of the theoretical maximum.

Before these probes were utilized in hybridization reactions with RNA, both null mDNA and RNA fractions were annealed to a vast excess of unlabeled, sheared rat DNA as described previously. The extent of [3H]DNA hybridization was determined by S1 nuclease digestion. Since the mDNA and null mDNA probes lost reactivity during the fractionation procedures, it was necessary to normalize the mDNA and null mDNA hybridization experiments to 100% tracer reactivity. This was done by dividing the DNA-RNA hybridization values at saturation by the fraction of mDNA or null mDNA which was hybridized to unlabeled DNA at DNA C51 values >5000.

**Hybridization of cDNA's to Poly(A)+ and Poly(A)− RNA's.** Hybrid-
zation reactions were performed, as described previously (13). The extent of hybridization was assayed by S1 nuclease digestion. The $R_{0.5}$ values were corrected for hybridization in 0.6 M Na+ (39). Hybridizations performed with the poly(A)− cDNA’s required a parallel set of duplicate RNA samples hydrolyzed in alkali to completely degrade the sequences were still complementary to rRNA. To control for this contamination of the probes by these rRNA sequences, parallel hybridizations were performed using rRNA in place of the experimental RNA’s in the hybridization reactions. These values were subtracted from those obtained with the experimental hybridizations prior to plotting the data.

Hybridization of [3H]DNA to Poly(A)− RNA’s. RNA excess hybridizations were carried out in tapered reaction vials in a final volume of 5 μl overlayed with sterile mineral oil to reduce evaporation. A constant ratio of RNA to [3H]DNA (single-copy [3H]DNA, 3H-null mDNA, or [3H]mDNA) was maintained in each vial, and the time of incubation was varied to obtain the desired $R_{0.5}$ values. Hybridization reactions were performed as described previously (13). To determine the percentage of duplex formation due to annealing of the probe to any contaminating DNA in the RNA preparations, controls were included that consisted of duplicate RNA samples hydrolyzed in alkali to completely degrade the RNA. $R_{0.5}$ values were corrected for hybridization in 0.6 M Na+. The extent of hybridization was assayed by S1 nuclease digestion.

RESULTS

Characterization of Midpregnant mDNA and Null mDNA. While saturation hybridization with single-copy DNA has been widely accepted as the method of choice in measuring the total complexity of RNA populations, it is clear that additivity experiments lack the sensitivity to detect subtle differences in complexities between different RNA populations. For this reason, midpregnant mDNA and null mDNA probes were isolated as described in “Materials and Methods” in order to increase the level of discrimination of the saturation hybridization assay. Prior to hybridization of the single-copy DNA probes to the RNA’s, it was necessary to demonstrate that the probes were not contaminated with repetitive DNA. To test this possibility, both probes were reacted with a vast excess of sheared, total rat DNA. The data in Chart 1 demonstrate that both probes are essentially free of repetitive sequences, as is the original single-copy [3H]DNA from which the probes were isolated. However, the extent of hybridization was only 60% for the fractionated probes as compared to 85% for the starting unique sequence DNA. This indicates that these probes lost reactivity during the fractionation procedures. The shift of the curve to the right ($C_{oD_{1/2}} = 2500$) when compared to the unfractionated probe ($C_{oD_{1/2}} = 1250$) suggests that a decrease in the size of the recycled probe has occurred, as well as loss of probe reactivity. Therefore, in order to normalize the data, all hybridizations were corrected to 100% tracer reactivity by dividing the observed saturation values of the null mDNA or mDNA reactions by 0.6, which was the extent of hybridization when hybridized to rat DNA.

Hybridization of a Single-Copy [3H]DNA Probe Depleted of Midpregnant Mammary Gland Poly(A)+ RNA Sequences. Since we have shown previously by saturation hybridization with unfractionated single-copy [3H]DNA that there are no detectable qualitative differences between the total poly(A)+ RNA’s from DMBA-induced tumor and midpregnant mammary glands, we prepared a sensitive probe for those sequences absent from the midpregnant RNA. This was done by 2 cycles of hybridization of single-copy DNA with midpregnant RNA. The DNA which remained single stranded is termed the midpregnant null mDNA. To insure that the number of counts of the [3H]DNA probes present in RNA-DNA hybrids was sufficient to permit accurate quantitation, a minimum of 10,000 cpm of probe DNA was present in all reactions so that >100 cpm of DNA would be in the RNA-DNA hybrids over the S1-resistant background. This probe hybridized with midpregnant RNA to a saturation value of approximately 3.0% (Chart 2) compared to 8.7% hybridization observed initially when the midpregnant RNA was hybridized to the unfractionated single-copy DNA. It appears that the presence of midpregnant RNA complementary sequences in the null tracer is due to a failure to bind all of the RNA-DNA hybrids during the HAP fractionation. Assuming that these residual sequences are representative of the entire set of midpregnant RNA’s, the presence of these sequences should not preclude use of this tracer to detect nonmidpregnant sequences. Reaction of the null mDNA with tumor poly(A)+ RNA gave a saturation value of 7.5% which is 2.5 times higher than the reaction of the null tracer with midpregnant RNA (Chart 2). Reaction of the probe with identical RNA samples hydrolyzed first with alkali in order to monitor potential DNA contamination resulted in virtually no hybridization. This demonstrates that the DNase treatment of the RNA samples effectively removed all of the contaminating DNA. A summary of the hybridization data is shown in Table 1.

The 4.5% difference between the saturation values obtained with tumor and midpregnant RNA’s when reacted with the null mDNA probe represents a complexity of 6.7 x 107 nucleotides (assuming asymmetrical transcription). This is equivalent to 9500 different 7-kb genes which are expressed in the tumor but not in the normal mammary gland. An average gene size of 7 kb was used for the following reason. Since total poly(A)+ RNA was utilized in these studies, most of the observed complexity is contributed by heterogeneous rRNA. The number average mRNA size in animal cells has been estimated to be about 1.4 kb. Since the primary rRNA transcripts are 2 to 6 times larger than the processed mRNA (12), using 7.0 kb as an average gene size appears to represent a valid assumption.
Therefore, this experiment suggests that the tumor poly(A)* RNA contains a set of sequences which are absent in the midpregnant RNA.


The reciprocal experiment involved the hybridization of a [3H]DNA probe, which was complementary to the RNA's expressed in the midpregnant gland (midpregnant mDNA), with homologous and heterologous RNA populations. The data from these experiments are shown in Chart 3. At saturation, the mDNA reacted with midpregnant RNA to an extent of 80%. In contrast, there was a decrease in the saturation value to roughly 50% when tumor RNA is reacted with the mDNA. An analysis of these data shown in Table 1 indicates that there are approximately 12,800 sequences in the midpregnant mDNA which do not react with the tumor RNA. This implies that there are some DNA sequences expressed in the midpregnant mammary gland but not in the tumor. While it appears that the hybridization of the mDNA with its homologous RNA has reached saturation, it is probable that this has not occurred for the heterologous reaction. It is possible, therefore, that the actual difference is less than that observed if the heterologous reaction has not gone to completion. However, these results indicate that there are detectable qualitative differences in the total poly(A)* RNA populations in the normal and neoplastic tissues.

Characterization of Poly(A)* RNA's. Poly(A)* RNA fractions were obtained from DMBA-induced mammary tumors and 14-day midpregnant mammary glands by oligo(dT)-cellulose chromatography as described in "Materials and Methods." Agarose-urea gel electrophoresis revealed that most of the mass in the poly(A)* fractions was 18S and 28S rRNA's. It was estimated by densitometric scans of the gels that these sequences account for 90 to 95% of the mass of the poly(A)* RNA's (data not shown). In order to determine whether the non-rRNA sequences of the poly(A)* fractions were simply deadenylated poly(A)* RNA's, which did not bind to oligo(dT)-cellulose under the conditions used or were actually unique

Table 1
Summary of mDNA and null mDNA hybridization reactions

<table>
<thead>
<tr>
<th>RNA</th>
<th>% of [3H]-null mDNA * hybridized</th>
<th>Complexity (nucleotides x 10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>No. of 7-kb genes not shared with midpregnant</th>
<th>% of [3H]-mDNA hybridized</th>
<th>Complexity (nucleotides x 10&lt;sup&gt;6&lt;/sup&gt;)</th>
<th>No. of 7-kb genes not shared with tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midpregnant</td>
<td>3.0</td>
<td>4.5</td>
<td>9,500</td>
<td>80.0</td>
<td>2.6</td>
<td>12,800</td>
</tr>
<tr>
<td>Tumor</td>
<td>7.5</td>
<td>11.0</td>
<td>9,500</td>
<td>50.0</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

* Represents the terminal saturation values obtained from the hybridization data presented in Charts 2 and 3. These values were normalized to 100% of their reactivity as indicated in "Materials and Methods."

a Ret single-copy DNA has a complexity of 1.9 x 10<sup>6</sup> nucleotide pairs (3). The null mDNA complexity equals that of total single-copy DNA (1.9 x 10<sup>6</sup>) less the complexity of the midpregnant mDNA (3.3 x 10<sup>6</sup> nucleotide pairs assuming asymmetrical transcription), or 1.5 x 10<sup>6</sup> nucleotide pairs. Hence, complexity = (% of [3H]-null mDNA hybridized) x (1.5 x 10<sup>6</sup>).

c No. of 7-kb genes = (complexity of null mDNA) x (0.045) x 7 x 10<sup>3</sup>.

d Complexity of midpregnant mDNA (3.3 x 10<sup>6</sup> nucleotide pairs) = 1.9 x 10<sup>6</sup> x 0.087 (% of hybridized [3H]-mDNA) x 2 (this value corrects for asymmetric transcription).

No. of 7-kb genes equals complexity of midpregnant RNA hybridized to probe (2.6 x 10<sup>6</sup>) less the complexity of tumor RNA reacted with the same probe (1.7 x 10<sup>6</sup>). This value (9 x 10<sup>6</sup>) was then divided by 7 x 10<sup>3</sup>.
poly(A)$^-$ RNA sequences, it was necessary to perform the following experiment.

Previously, we have reported that the poly(A)$^+$ RNA populations from the tumors and the normal gland contained 5.5 to 5.7% poly(A) as determined by hybridization with $[^3H]$poly(U) (36). The poly(A) content of both poly(A)$^-$ RNA fractions was 0.03 to 0.04% which amounts to a 185-fold reduction. This indicates that there are probably poly(A)$^+$ sequences present in this fraction which possess poly(A) tracts too short to permit binding to oligo(dT)-cellulose. It has been reported that poly(A) tracts shorter than 20 to 30 residues are not efficiently bound by oligo(dT)-cellulose (8).

In order to estimate the possible sequence overlap of poly(A)$^-$ and poly(A)$^+$ RNA's, cDNA probes synthesized from the poly(A)$^-$ RNA's were used in hybridization reactions with poly(A)$^-$ and poly(A)$^+$ RNA's. The use of random priming for the cDNA synthesis insures that both the 3' and the 5' ends of the template RNA's are copied. This allows a more accurate measurement of the complexity of contaminating poly(A)$^+$ RNA's. Results of the hybridization of midpregnant and tumor poly(A)$^-$ cDNA to their respective poly(A)$^-$ and poly(A)$^+$ RNA's are shown in Chart 4. The kinetics of hybridization of the cDNA's with their poly(A)$^-$ RNA templates revealed a complex population of hybridizable RNA's which appeared to have more than one frequency class. The extent of hybridization was approximately 80% for both tumor (Chart 4A) and midpregnant (Chart 4B) poly(A)$^-$ RNA's. Further inspection of the hybridization data (Chart 4B) reveals that the midpregnant poly(A)$^-$ RNA has an abundant component which is not present in the tumor poly(A)$^-$ RNA. This component was shown to be composed mainly of the milk protein (casein and $\alpha$-lactalbumin) sequences which account for 40 to 50% of the mass of the poly(A)$^+$ RNA from the midpregnant gland (36). Titration hybridization of the midpregnant poly(A)$^-$ RNA with a specific 15S casein cDNA probe ($\alpha$ and $\beta$) revealed that 10 to 15% of these sequences are found in the poly(A)$^-$ fraction (data not shown). This would account for a high percentage of the mass of the non-rRNA poly(A)$^-$ RNA sequences but would contribute little to the total complexity in this RNA fraction. The values for the hybridization to the homologous poly(A)$^+$ RNA's were approximately 20% for both the tumor and midpregnant RNA's.

While it appears that the hybridization of the cDNA's to both poly(A)$^-$ RNA fractions went to completion, it is not clear whether the reactions with the poly(A)$^+$ RNA's reached saturation. Low-frequency, high-complexity poly(A)$^+$ RNA's contaminating the poly(A)$^-$ fractions from which the cDNA was synthesized would be represented at extremely low levels in the cDNA probe, making it difficult to achieve saturation when hybridized to the poly(A)$^+$ RNA's. Therefore, the hybridization value of 20% in Chart 4, A and B, must be taken as the minimum level of contamination of the poly(A)$^-$ RNA's. These data indicate that there is a complex class of poly(A)$^-$ sequences; however, there is significant contamination with poly(A)$^+$ RNA sequences which is difficult to quantitate precisely.

**Hybridization of Poly(A)$^-$ RNA's to $[^3H]$DNA.** Having shown that there is a unique set of poly(A)$^-$ RNA sequences in both the tumors and normal mammary gland that are not overlapping with poly(A)$^+$ RNA's, we used saturation hybridization with single-copy DNA to determine if there were qualitative differences between the 2 poly(A)$^-$ RNA populations. The isolation and characterization of the unique sequence DNA probe have been described previously (Chart 1). Both tumor and midpregnant poly(A)$^-$ RNA's gave saturation values of 3.0% at high $R_0t$ values (>$500,000$; Chart 5).

In order to determine the degree to which the poly(A)$^-$ RNA sequences were shared between the 2 tissues, mixtures of the 2 RNA's were hybridized to the single-copy DNA probe. The mixture of the RNA's saturated at 3.0% which was the same as the 2 RNA's alone. This demonstrates that the poly(A)$^-$ RNA's contain totally overlapping sequences, within the limits of de-
correction of this technique, which would probably not detect differences of less than 10 to 20%. These data have not been corrected for the hybridization of the contaminating poly(A)+ RNA sequences to the [3H]DNA probe which could account for as much as 15 to 25% of the 3% hybridization observed at saturation. This contamination would make it more difficult to detect differences in the poly(A)− RNA populations, since we have shown previously that we could not find qualitative differences using this technique between the poly(A)− RNA's isolated from the tumors and midpregnant gland. With these reservations in mind, we conclude that the majority of the poly(A)− RNA sequences are held in common between the neoplastic and the normal tissues.

**DISCUSSION**

By using fractionated single-copy DNA hybridization probes, we have detected a complex set of sequences which are not shared between the poly(A)+ RNA populations isolated from DMBA-induced mammary tumors and normal mammary tissue. In addition, both tissues possess a unique class of poly(A)− RNA's. However, saturation hybridization with unfractionated single-copy DNA indicates that most of these sequences are shared between the normal and neoplastic tissues.

Our initial investigation using cDNA complexity analysis and single-copy DNA saturation hybridization suggested, at the level of discrimination afforded by these techniques, that no qualitative differences existed between total poly(A)+ RNA's isolated from neoplastic and normal mammary tissue (36). This discrepancy with our present results can be explained as follows. DNA hybridization provides an excellent technique for analyzing the complexity and frequency changes in the abundant and moderately abundant RNA frequency classes. Both Van Ness and Hahn (37) and Savage et al. (34) also report that cDNA probes can be used to make valid qualitative and quantitative comparisons of the complex, infrequent class mRNA's when used to analyze polysomal poly(A)+ RNA populations. However, we found that in using this method we consistently underestimated the complexity of the infrequent or scarce RNA's when total cellular poly(A)+ RNA's (nuclear and cytoplasmic RNA) were analyzed. Using cDNA hybridization, the total complexity of the poly(A)+ RNA's from the neoplastic and normal tissues was estimated to be $3.0 \times 10^7$ to $4.4 \times 10^7$ nucleotide pairs, while saturation hybridization with [3H]DNA gave values of $2.8 \times 10^8$ to $3.3 \times 10^8$ nucleotide pairs. A similar result has also been reported by other investigators (21, 29). The cDNA complexity analysis may result in an underrepresentation of the high-complexity, infrequent mRNA's. Abundant and moderately abundant cytoplasmic RNA's contribute the bulk of the mass but a small percentage of the complexity of cellular mRNA. The cDNA's synthesized from these cytoplasmic, mature mRNA's are essentially full length and represent both the 3' and 5' ends of these RNA's. There is evidence that the large, high-complexity mRNA's are less efficient templates for cDNA synthesis (25). The reverse transcriptase may fall off the larger mRNA templates, and therefore, the resulting cDNA may only be representative of the 3' end of these RNA's. Thus, the nucleotide complexity at the 5' ends of these molecules is not represented in the cDNA. The infrequent mRNA's may, therefore, be underrepresented in the cDNA population, resulting in an underestimate of the complexity of this class and making it more difficult to detect changes that occur between 2 different RNA populations.

By fractionating the single-copy DNA into null mRNA and mRNA components, the level of discrimination may be increased by more than 10-fold (7, 16). Saturation hybridization with the null mRNA probe revealed a 4.5% difference between the saturation values observed with the tumor and midpregnant RNA's. The nucleotide complexity of this set of sequences which is found in tumor but not in midpregnant RNA is $6.7 \times 10^7$ nucleotides. This is equivalent to 25% of the total complexity of the tumor RNA observed previously. This represents a change of 3.5% in the transcription of the single-copy genome, which is probably why we were unable to detect this change using unfractionated [3H]DNA. It must be kept in mind that this value of 3.5% of the single-copy genome is actually double the experimentally observed value of 1.75% which was obtained since all hybridization values were multiplied by 2 to correct for asymmetrical transcription. A change of this magnitude is most probably at the limit of detection using unfractionated [3H]DNA in additivity experiments.

Experiments utilizing a midpregnant mRNA probe provided further evidence for changes in single-copy DNA expression between the 2 tissues. Roughly 30% of the RNA sequences in the midpregnant gland were absent from the tumor. This set of sequences had a complexity of $8.9 \times 10^7$ nucleotides. This represents a change in transcription of 4.5% of the single-copy rat genome. Since saturation was not achieved when the tumor RNA was reacted with the mRNA (Chart 3), the actual value may be much lower than that which was observed. In addition, a dramatic shift in the frequencies of certain RNA species between the 2 tissues might possibly appear as a qualitative change and tend to exaggerate the differences detected. In order to explain the observed differences in terms of frequency changes, it would, however, require that the concentration of RNA molecules responsible for the change fall to less than 0.1 copy/cell (11). With this reservation in mind, we conclude that there are distinct sets of RNA's present in one tissue and not another, even though the actual difference may be smaller than we observed. Using a similar method, Grady et al. (11) demonstrated that there was an 18% difference in the transcription of nonrepetitive DNA between normal and regenerating rat liver. This difference was not seen in the mRNA's of these tissues, when an unfractionated [3H]DNA probe was used. Similarly, Ernst et al. (7) have reported that distinct sets of mRNA's exist between sea urchin embryos and adult tissues. Previous reports indicated that there was no difference in mRNA's at different sea urchin developmental stages (14, 20, 40). This is in contrast to the findings of Moyzis et al. (27), who used a similar approach but did not detect tumor-specific mRNA's in a chemically transformed hamster cell. It is not possible to conclude whether the sets of RNA sequences that are not shared between the DMBA-induced mammary tumors and the normal mammary gland represent tissue-specific, structural genes the expression of which is necessary for the maintenance of either the normal or transformed phenotype.

It appears that, when the mRNA populations of different cell types of the same lineage are compared (i.e., normal versus transformed cells), the most striking difference observed is changes in the relative abundances of mRNA's (7, 15, 22, 23, 36). However, these observations have been based on the techniques of cDNA hybridization or saturation hybridization...
with unfractionated [\textsuperscript{3}H]DNA, which lack the discrimination to detect subtle qualitative changes between RNA populations. Thus, saturation hybridization studies with fractionated probes will be necessary to determine if there are qualitative differences between polyosomal RNA populations in different cells of the same lineage.

Even though the majority of these sequences may not become functional mRNA's, it is conceivable that these unique transcripts could perform some regulatory function in the nucleus (6). It is also possible that these sequences are transcribed and then rapidly turned over in the nucleus (3). Positive identification and cloning of some of these "tumor-specific" sequences will be required to further study their biological significance. It seems likely, however, that some of the scarce and possibly moderately abundant RNA sequences could become functional mRNA's. There is evidence that there are organ-specific proteins coded for by scarce mRNA's. Galau et al. (9) have reported that in the liver there are several enzymes the function of which is important to the liver in physiological terms but which appear to require for maintenance only 1 to 10 mRNA molecules/liver cell. Thus, it is probable that many regulatory proteins that are necessary for the expression of a specific phenotype are encoded by scarce mRNA's. Thus, the differences in cellular phenotype may be brought about by a relatively small number of specific sequences in conjunction with the regulation of the abundances of sequences that are held in common between the 2 tissues.

Further investigation of the RNA populations from DMBA-induced tumors and normal mammary tissue revealed a complex class of poly(A)\textsuperscript{+} RNA sequences in both tissues. Hybridization of each of these RNA populations to unfractionated [\textsuperscript{3}H]DNA yielded a saturation value of 3% of the nonrepetitive DNA. The complexity of this class of RNA's was approximately 5.7 x 10\textsuperscript{7} nucleotides. This value has not been corrected for the contamination by the poly(A)\textsuperscript{+} RNA sequences. These results are in agreement with reports from Chikaraiski (5) and Van Ness et al. (38), who have reported that a complex class of poly(A)\textsuperscript{+} RNA is present in both rat and mouse brain. The high complexity of the poly(A)\textsuperscript{+} RNA class indicates that much of the complexity probably is derived from mRNA. From these analyses, it is not possible to determine if there is a unique class of functional polysomal poly(A)\textsuperscript{+} mRNA's. Bantle et al. (2) reported a complete overlap between poly(A)\textsuperscript{+} and poly(A)\textsuperscript{−} RNA's in mouse liver. However, their analysis of nuclear poly(A)\textsuperscript{+} RNA's did not demonstrate a complete overlap of these sequences.

Additivity experiments indicated that the great majority of the poly(A)\textsuperscript{−} RNA's were also held in common between the tumors and the normal gland. The presence of contaminating poly(A)\textsuperscript{+} RNA species (15 to 25%) in the poly(A)\textsuperscript{−} fraction would make it difficult to determine if there were qualitative differences in these RNA samples. Use of fractionated [\textsuperscript{3}H]DNA probes to detect differences would necessitate removing most of the contaminating poly(A)\textsuperscript{+} RNA's by techniques other than oligo(dT)-cellulose chromatography prior to performing these recycling experiments. Van Ness et al. (11) have reported that chromatography of RNA fractions on benzoylated cellulose effectively separates 98 to 99% of the poly(A)\textsuperscript{+} RNA's from the poly(A)\textsuperscript{−} fraction. Other investigators using oligo(dT)-cellulose to separate poly(A)\textsuperscript{+} and poly(A)\textsuperscript{−} RNA's have found a class of mRNA's whose members are both poly(A)\textsuperscript{+}, poly(A)\textsuperscript{−}, or contain oligoadenylate tracts and encode common poly(A)\textsuperscript{−} and poly(A)\textsuperscript{+} mRNA products (5, 18). Milcerek et al. (26) demonstrated that both poly(A)\textsuperscript{−} and poly(A)\textsuperscript{+} mRNA's appear in the cytoplasm of HeLa cells with approximately parallel kinetics. Hence, a portion of the poly(A)\textsuperscript{−} mRNA is not derived by the loss of poly(A)\textsuperscript{+} in the cytoplasm from poly(A)\textsuperscript{+} mRNA.

Homologous hybridizations performed between poly(A)\textsuperscript{−} RNA's and their respective cDNA's (Chart 4) suggested that this complex class of RNA's possessed different frequency classes, which is analogous to the poly(A)\textsuperscript{+} RNA's. However, with the exception of histone mRNA's, the functions of the poly(A)\textsuperscript{−} RNA's in mammalian cells are not known. Future studies in this area will require the positive identification of proteins coded for by poly(A)\textsuperscript{−} RNA's. In addition, it will be necessary to elucidate the pathways by which poly(A)\textsuperscript{+} and poly(A)\textsuperscript{−} RNA's are processed and transported out of the nucleus.

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Tumor-specific Polyadenylated RNA’s from 7,12-Dimethylbenz(a)anthracene-induced Mammary Tumors Revealed through Hybridization with Fractionated Single-Copy DNA

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