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Gene Expression in Normal and Neoplastic Mammary Tissue†

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ABSTRACT: The techniques of molecular hybridization and cell-free translation have been utilized to analyze total cellular poly(A^+) RNA populations from hormone-dependent, 7,12-dimethylbenz[*a*]anthracene (DMBA) induced mammary adenocarcinomas and from normal 14-day midpregnant mammary glands in Sprague-Dawley rats. These studies were designed to elucidate any marked changes in gene expression that might occur in these primary hormone-dependent mammary tumors, resulting in a deviation from normal hormone-regulated growth and differentiation. Homologous and heterologous hybridizations of poly(A^+) RNAs from DMBA-induced mammary tumors and normal mammary glands to complementary DNAs (cDNAs) revealed no detectable qualitative differences between the two RNA populations. However, there were significant quantitative differences in the abundancies of certain poly(A^+) RNA sequences. Specifically, the milk protein sequences were reduced 100-fold in the tumor when compared to the normal midpregnant mammary gland. Saturation hybridization of the poly(A^+) RNAs to ^{3}H-labeled single copy DNA revealed that both RNA populations annealed to 7.5% of the single copy haploid genome, as did mixtures of the two RNA populations, which again indicated that the great majority of the poly(A^+) RNA sequences were shared between the two tissues. Analysis of cell-free translation products by two-dimensional gel electrophoresis indicated quantitative and possibly qualitative differences in the proteins specified by the two mRNA populations. These data suggest that large alterations in gene expression are not required for the altered function found in the neoplastic tissue and that differences in relative abundancies of specific RNAs may provide a mechanism by which the concentration of specific proteins and ultimately the expression of the transformed phenotype may be regulated.

The same hormones that are important regulators of growth and differentiated function in the normal mammary gland are also necessary for the growth of hormone-dependent mammary adenocarcinomas. Administration of the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA)[1] to female rats results in the induction of mammary adenocarcinomas. Approximately 80–90% of these tumors are hormone dependent, as demonstrated by endocrine ablation and hormone replacement (Huggins & Bergenstal, 1952). Hormonally responsive DMBA-induced mammary tumors may exhibit differentiated functions that are characteristic of the normal mammary gland. Previous studies in our laboratory utilizing a specific cDNA hybridization probe revealed that casein mRNA was present in 70% of the DMBA-induced mammary tumors that were assayed. However, these casein mRNA levels were only 1–10% of those observed in 8-day lactating rat mammary tissue (Rosen & Socher, 1977).

The purpose of this study was to utilize the techniques of molecular hybridization and cell-free translation to compare the total poly(A^+) RNA populations in the DMBA-induced mammary tumor and the normal mammary gland. This approach was based on the rationale that changes in the patterns of RNA synthesis or processing lead to qualitative as well as quantitative alterations in the pool of mRNA sequences translated into proteins that ultimately modulate cellular function and phenotype. Molecular hybridization employing both cDNA and unique sequence DNA has been used to measure the number of poly(A^+) RNA sequences present in the cells in question, while cell-free translation allowed a comparison of the proteins that are specified by the mRNA populations in the tumor and the normal mammary gland.

Several previous studies have employed molecular hybridization to compare poly(A^+) RNA populations in different tissues (Hastie & Bishop, 1976; Chikaraishi et al., 1978), during different stages of differentiation (Paterson & Bishop, 1977; Affara et al., 1977) and, more importantly for this study, in normal and transformed cells (Getz et al., 1977; Kuo et al., 1976; Williams et al., 1977; Moyzias et al., 1976). However, to date, the comparisons of poly(A^+) RNA populations between normal and transformed cells have been done in cultured cells or in transplantable tumors. Since neoplastic progression is known to be a characteristic of both transformed cell lines and transplantable tumors, it is likely that the pattern of gene expression in these cells may be different from that of the

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[1] Abbreviations used: DMBA, 7,12-dimethylbenz[*a*]anthracene; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Na_2EDTA, disodium ethylenediaminetetraacetic acid; cDNA, complementary DNA; RNA_{mp} and RNA_t, poly(A^+) RNA from 14-day midpregnant mammary glands and DMBA-induced mammary tumors, respectively; $cDNA_{mp}$ and $cDNA_t$, complementary DNAs synthesized by using poly(A^+) RNA from 14-day midpregnant mammary gland and DMBA-induced mammary tumors as templates; C_0t, DNA concentration in moles of nucleotide per liter × time in seconds; R_0t, RNA concentration in moles of nucleotide per liter × time in seconds.

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original primary tumor. In the present study, the techniques of molecular hybridization and cell-free translation have been utilized in carcinogen-induced primary tumors, to determine if the pattern of activated and repressed genes has undergone significant changes compared to normal tissues.

These techniques have been employed to answer several different questions: (1) What are the complexities and abundancy class compositions of these two RNA populations? (2) To what extent are poly(A^+) RNA sequences held in common between the RNA populations from the neoplastic and normal tissue, and is there a class of tumor-specific poly(A^+) RNA sequences? (3) Are there changes in the frequencies of different poly(A^+) RNAs between the tumor and the normal mammary gland, and, specifically, how are the levels of the milk protein sequences changed? (4) If there are quantitative or qualitative differences between the two poly(A^+) populations, how is this reflected in the proteins that are synthesized in vitro from these RNA sequences in the neoplastic and normal tissue?

Experimental Procedures

Induction of Mammary Tumors. Mammary adenocarcinomas were induced in 50-day-old female Sprague-Dawley rats by a series of four weekly intubations with 5 mg of DMBA dissolved in sesame oil (Huggins & Bergenstal, 1952). Tumors appeared 6–14 weeks following the first treatment. 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 (Huggins & Bergenstal, 1952; Bradley et al., 1976). Therefore, only rapidly growing tumors that appeared during this time were utilized in these experiments. To ensure that these tumors were adenocarcinomas, histological examination of each tumor was performed. Animals were sacrificed by cervical dislocation, and the dissected tumors were washed in cold saline solution and quick frozen in liquid N_2. Normal mammary tissues were obtained from 14-day midpregnant Sprague-Dawley rats. The mammary glands were removed and quick frozen, as described previously (Rosen et al., 1975).

Isolation and Characterization of Poly(A^+) RNA. Total nucleic acids were extracted from quick-frozen DMBA-induced mammary tumors and 14-day midpregnant, mammary glands in 20 volumes (v/w) of a phenol–chloroform–NaDodSO$_4$ solution at pH 8.0 (Rosen et al., 1975). The nucleic acid extracts were treated with proteinase K as described by Guyette et al. (1979), and DNA and small RNA molecules were removed by three extractions with 3 M sodium acetate, pH 6.0, at 4 °C.

Poly(A^+) RNA was isolated by two passages over oligo(dT)-cellulose, as described previously (Rosen, 1976). Analytical agarose–urea gel electrophoresis was performed on 2% agarose gels (Bio-Rad) in 6 M urea and 0.025 M citric acid, pH 3.5 (Rosen et al., 1975; Woo et al., 1975). Gels were stained for 30 min in a 5 μg/mL ethidium bromide–50 mM Tris, pH 7.5, solution, destained for 15 min in water, and visualized on an ultraviolet illuminator using short-wave UV. The amount of poly(A) present in the oligo(dT)-bound nucleic acid was estimated by hybridization with [^{3}H]poly(U), as described previously (Kaufman & Gross, 1975). The number-average size of both poly(A^+) RNA populations was determined by hybridization with [^{3}H]poly(dT) across 8 to 30% (w/v) linear sucrose gradients containing 70% formamide as described by Monahan et al. (1976).

Synthesis and Characterization of cDNA. High specific activity, full-length cDNAs were synthesized from the poly(A^+) RNA fractions from the DMBA-induced mammary tumors and the 14-day midpregnant mammary glands following the procedure of Matusik & Rosen (1978). The [^{3}H]cDNA had a specific activity of 1.5×10^7 cpm/μg. The S_1 resistant backgrounds for both cDNA preparations were approximately 1.5%. Size determination and fractionation of the cDNAs were accomplished by alkaline sucrose gradient centrifugation (Studier, 1965). Complementary DNA greater than 250 nucleotides in length was utilized in all subsequent hybridization studies.

Isolation, Nick Translation, and Characterization of Unique Sequence DNA. DNA was isolated from rat liver by extraction with chloroform–isoamyl alcohol–NaDodSO$_4$, as described in detail elsewhere (Rosen et al., 1973). The DNA was then sheared at 16000 psi in a French pressure cell press (Aminco) to a single-stranded fragment length of approximately 400 nucleotides, denatured in alkali, neutralized, and reannealed to a C_0t of 600. Single-stranded DNA was isolated on hydroxylapatite and annealed a second time to a C_0t of 600. The single-stranded DNA reisolated on hydroxylapatite was reannealed to a C_0t of 50000 and nick translated, with the omission of DNase, by the method of Mackey et al. (1977). In order to remove hairpin structures from the nick-translated DNA, we again isolated single-stranded DNA on hydroxylapatite. Under the conditions employed, the [^{3}H]DNA had a specific activity of 4.0×10^6 cpm/μg and a mean length of 300 nucleotides, as determined by alkaline sucrose gradient centrifugation (Studier, 1965). Approximately 2000 cpm of single-copy [^{3}H]DNA was then hybridized to an excess of sheared rat liver DNA (Rosen et al., 1973). The extent of both [^{3}H]DNA hybridization and DNA–DNA renaturation was determined by hydroxylapatite chromatography (Rosen et al., 1973).

Hybridization of cDNA to Poly(A^+) RNA. Hybridizations were performed as described by Harris et al. (1975). Each vial contained a minumum of 1500 cpm of [^{3}H]cDNA (DMBA tumor or 14-day midpregnant mammary gland), 40 μg/mL hen oviduct RNA, and varying amounts of poly(A^+) RNA from either DMBA-induced mammary tumors or 14-day midpregnant mammary glands. Following heat denaturation at 100 °C for 1 min, incubations were carried out for 48 h at 68 °C, and the reaction was terminated by freezing at −80 °C. The extent of hybridization was assayed by S_1 nuclease digestion. The R_0t values were corrected for hybridization in 0.6 M Na^+ (Wetmur & Davidson, 1968). The hybridization data were analyzed by using a computer program that determined the best fit for an ideal pseudo-first-order curve and also determined the apparent $R_0t_{1/2}$ values (Monahan et al., 1976).

Hybridization of Unique Sequence [^{3}H]DNA to Poly(A^+) RNAs. RNA excess hybridizations were carried out in tapered reaction vials in a final volume of 5 μL, overlayered with sterile mineral oil to reduce evaporation. A constant ratio of RNA to [^{3}H]DNA was maintained in each vial, and the time of incubation was varied (0.5–168 h) to obtain the desired R_0t values. Hybridization reactions were performed as described previously (Harris et al., 1975). To determine the percentage of duplex formation due to annealing of the probe to any contaminating DNA in the poly(A^+) RNA preparation, controls were included that consisted of duplicate poly(A^+) RNA samples hydrolyzed in alkali to completely degrade the RNA. R_0t values were corrected for hybridization in 0.6 M Na^+ (Wetmur & Davidson, 1968).

Cell-Free Translation of Poly(A^+) RNA. Poly(A^+) RNAs obtained from DMBA-induced mammary tumors and 14-day midpregnant mammary glands were translated in the nu-

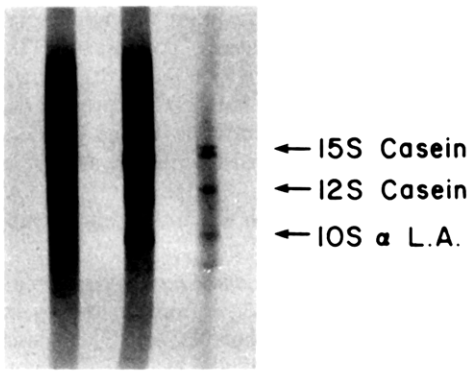

FIGURE 1: Agarose–urea gel electrophoresis analysis of poly(A^+) RNAs. Five micrograms of tumor (A) and 14-day midpregnant (B) poly(A^+) RNAs, which were purified by two passages over oligo(dT)-cellulose, were analyzed on 2% agarose–6 M urea gels as described under Experimental Procedures. Milk protein mRNA standards (C) were from an 8-day lactating mRNA preparation specifically enriched for these milk protein sequences.

clease-treated rabbit reticulocyte translation system using a modification of the method of Pelham & Jackson (1976). The lysate and all reagents were supplied in a [^{35}S]methionine reticulocyte lysate translation kit purchased from New England Nuclear.

Analysis of Translation Products by Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis of the in vitro translation products was performed according to the method of O'Farrell (1975). Separation of polypeptides in the first dimension was performed on 4% acrylamide isoelectric focusing gels containing 2% Ampholenes (1.6% pH range 3.6 and 0.4% pH range 3.5–10). To determine the pH gradient of the isoelectric focusing gels, a gel lacking a protein sample was cut into 4-mm sections and placed in 2 mL of degassed H_2O. After the solution was shaken for 15 min, the pH was measured on a pH meter. The second dimension employed the discontinuous $NaDodSO_4$ gel system described by Laemmli (1970). After electrophoresis, the gel was stained with Coomassie blue in order to visualize the molecular weight standards. The radioactive proteins were visualized by fluorography using the method of Bonner & Laskey (1974), with preflashed Kodak X-omat film to ensure better quantitation of the protein spots (Laskey & Mills, 1975).

Results

Isolation and Characterization of RNAs. Total cellular poly(A^+) RNA was isolated from pooled DMBA-induced mammary tumors and 14-day midpregnant tissue, as described under Experimental Procedures. In order to minimize hybridization artifacts due to factors such as extensive RNA degradation or preferential extraction of poly(A^+) RNA from one tissue when compared to the other, both RNA populations were characterized by size and percent poly(A) content. The percentage of poly(A^+) RNA isolated from both preparations following two passages over oligo(dT)-cellulose was approximately 2.0% of total RNA in both cases. Both tumor and normal poly(A^+) RNA populations were analyzed on agarose–urea gels. As shown in Figure 1, the poly(A^+) RNA from the 14-day midpregnant mammary gland displayed, over a background of heterogeneously sized mRNAs, the characteristic 15S casein doublet, the 12S casein mRNA band, and the 10S α-lactalbumin mRNA. The RNA from the DMBA-induced tumor did not display discrete milk protein mRNA bands but instead a heterogeneous distribution of poly(A)-containing RNA. Both samples were estimated to contain approximately 20% ribosomal RNA by densitometric scans.

Table I: Characterization of DMBA-Induced Mammary Tumor and Midpregnant Poly(A)-Containing RNAs and Their cDNAs

	nucleotides[a] (no. av)	% poly(A)[b]
tumor mRNA	950	5.5
tumor cDNA	850	
midpregnant mRNA	1320	5.7
midpregnant cDNA	1150	

[a] The number average nucleotide lengths for the poly(A^+) RNAs were determined by hybridization with [^{3}H]poly(dT) across formamide–sucrose gradients. Average lengths for the cDNAs were determined by alkaline–sucrose gradient centrifugation. [b] Percent poly(A) content was determined by hybridization with [^{3}H]poly(U).

The number-average polynucleotide chain length of each poly(A^+) RNA preparation was determined by formamide gradient centrifugation followed by hybridization of each gradient fraction with [^{3}H]poly(dT). The poly(A^+) RNAs sedimented with a broad range of *s* values. Employing tRNA and several mRNAs (ovalbumin, three casein, and α-lactalbumin mRNAs) as standards, it was possible to construct a standard curve from which the nucleotide lengths of RNA species in each gradient fraction could be calculated by using the relationship described by Spirin (1963). Cumulative plots of the fraction of total poly(A^+) RNA molecules vs. nucleotide length gave values of 950 and 1320 nucleotides for the tumor and midpregnant poly(A^+) RNAs, respectively (Table I). As shown in Table I, the RNA preparations contained 5.5 and 5.7% poly(A) sequences for the tumor and the normal mammary gland, respectively, as determined by hybridization to [^{3}H]poly(U). These data also suggested that both preparations had approximately the same amount of rRNA contamination, assuming that the average poly(A) tail lengths of both RNA populations were similar. These results indicated that both poly(A^+) RNA populations were isolated without extensive degradation and that both preparations were equivalent with respect to their poly(A) contents.

Characterization of [^{3}H]cDNA and ^{3}H-Labeled Unique Sequence DNA Hybridization Probes. The hybridization probes employed in this study were either cDNAs synthesized from poly(A^+) RNA templates isolated from DMBA-induced mammary tumors and 14-day midpregnant glands or nick-translated unique sequence DNA. Accurate quantitation of hybridization experiments depends upon a number of parameters, among which are the size of the radioactive probes (Wetmur & Davidson, 1968) and, in the case of unique sequence DNA, the absence of repeated sequence DNA contamination. Using alkaline sucrose gradient centrifugation and appropriate standards, we found the average nucleotide lengths for the tumor and midpregnant cDNAs to be 850 and 1150 nucleotides, respectively (Table I). To avoid hybridization artifacts due to very short cDNAs, only cDNAs >250 nucleotides were employed in subsequent hybridization experiments. The nick-translated [^{3}H]DNA had an average size of 300 nucleotides as determined by alkaline gradient centrifugation. In this case, only [^{3}H]DNA molecules >100 nucleotides in length were utilized for subsequent RNA excess hybridizations.

Hybridization of the [^{3}H]DNA probe to a large excess of unlabeled, sheared, total DNA (300–400 nucleotides) indicated that the single-copy probe was essentially free of repetitive DNA sequence contamination. Whereas approximately 30% of the driver DNA reassociated with kinetics characteristic

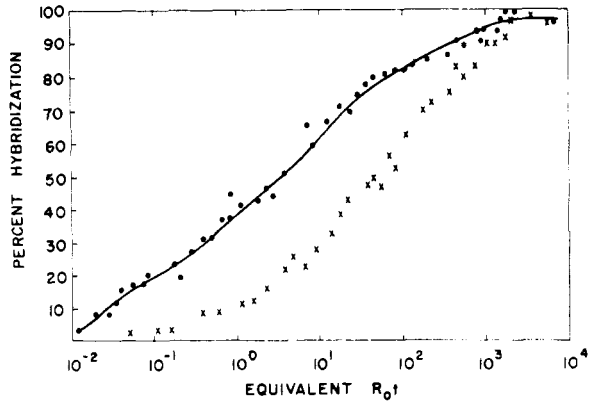

FIGURE 2: Kinetics of hybridization of poly(A^+) RNAs from DMBA-induced mammary tumors and 14-day midpregnant mammary glands to $cDNA_{mp}$. The concentration of poly(A^+) RNA for the homologous reaction (●) ranged from 0.0031 to 3.1 mg/mL. For the heterologous reaction (×), the tumor poly(A^+) RNA concentrations ranged from 0.13 to 2.7 mg/mL. The amount of $cDNA_{mp}$ was 0.12 ng. Other details are described under Experimental Procedures.

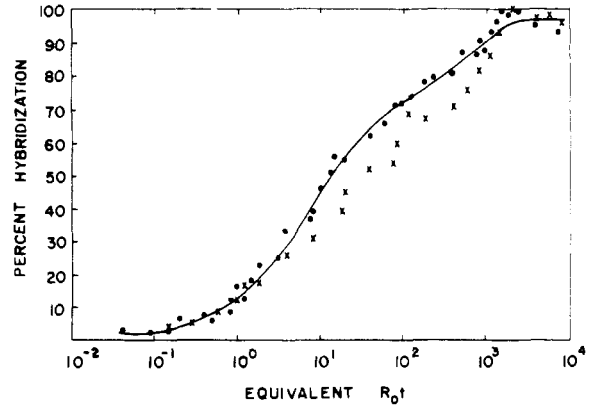

FIGURE 3: Kinetics of hybridization of poly(A^+) RNAs from DMBA-induced mammary tumors and 14-day midpregnant mammary glands to $cDNA_t$. The concentration of poly(A^+) RNA for the homologous reaction (●) ranged from 0.1 to 2.7 mg/mL. For the heterologous reaction (×), the midpregnant poly(A^+) RNA concentration ranged from 0045 to 3.1 mg/mL.

of repetitive sequence DNA, the nonrepetitive [^{3}H]DNA tracer remained >95% single stranded at a C_0t value of >50. The [^{3}H]DNA tracer reassociated with a rate constant of 4.5 × 10^{-4} L mol^{-1} s^{-1} and hybridized to a maximum of 85% as determined by hydroxylapatite chromatography (data not shown). Similar values for the proportion of nonrepetitive DNA and its reassociation constant have been reported previously for rat DNA (Holmes & Bonner, 1974; Kaplan et al., 1978). Before using the [^{3}H]DNA probe in saturation hybridization studies, it was necessary to remove the S_1 resistant hairpin loop structures that comprised approximately 20% of the nick-translated probe. This was accomplished by reisolation of single-stranded [^{3}H]DNA on hydroxylapatite. Following this procedure, the zero time S_1 resistance of the probe was 2.5%. This value was subtracted from the data prior to plotting.

Hybridization of Poly(A^+) RNA to cDNA. The sequence complexity of an unknown RNA population can be determined by a comparison of its $R_0t_{1/2}$ value with that of a kinetic standard of known sequence complexity. The kinetic standard chosen for this work was the homologous hybrid of 15S casein mRNA with its respective cDNA. Since the kinetics of hybridization between mRNA and cDNA are affected by the size of the cDNA, the casein cDNA preparation was fractionated on alkaline sucrose gradients, and only fractions greater than 600 nucleotides were used. By use of this fractionated cDNA probe, >95% hybridization was achieved with a $R_0t_{1/2}$ value of 1.1 × 10^{-2} mol·s L^{-1} under hybridization conditions identical with those employed for the complexity analysis. To compare the poly(A^+) RNA populations from the DMBA-induced mammary tumors and the normal mammary gland, the complexity and frequency distributions of both RNA fractions were analyzed by homologous hybridizations to their respective cDNA copies. Under conditions of RNA excess, the rate of hybridization reflects the complexity and frequency of the sequences within the RNA population (Bishop et al., 1974). The data obtained in hybridization experiments between the tumor and midpregnant cDNAs and their homologous poly(A^+) RNAs are shown in Figures 2 and 3. The hybridizations occurred over greater than 5 log units, which indicated that different RNA classes existed that could be separated on the basis of their sequence abundance. Computer analysis of the data indicated that for both homologous hybridizations a hybridization curve containing three different RNA classes could be generated and provided a statistical best fit for the data. The numbers of different sequences (1320 nucleotides long) in the three abundance classes were approximately 7, 340, and 9250 for the $cDNA_{mp}$–RNA_{mp} homologous hybridization, giving a total number of 9600 different sequences. For the $cDNA_t$–RNA_t homologous hybridization, the numbers of different 950-nucleotide-long sequences in the three abundance classes were 34, 740, and 14 500 for a total of 15 000 different sequences (Table II). Using an analysis of variance and data from three different experiments, we found that the number of poly(A^+) RNA sequences in the tumor was not significantly different from the number of sequences found in the midpregnant poly(A^+) RNA population.

Table II: Sequence Complexity of Total Poly(A)-Containing RNA in DMBA-Induced Mammary Tumor and Midpregnant Mammary Gland[a]

tissue	abundance class	$R_0t_{1/2}$ obsd[b]	fraction of total	$R_0t_{1/2}$ corrected[c]	no. of diff sequences[d]
midpregnant mammary gland	1	0.17	0.30	0.04	7
	2	5.7	0.40	1.8	339
	3	293.0	0.24	49.2	9 245
DMBA-induced mammary tumor	1	1.1	0.22	0.19	34
	2	10.9	0.45	3.9	735
	3	332.0	0.29	77.0	14 528

[a] Analyses of hybridization kinetics of data shown in Figures 2 and 3. [b] Observed $R_0t_{1/2}$ values were determined by computer analysis of the hybridization data. [c] $R_0t_{1/2}$(obsd) × fraction of total (% cDNA) as in Bishop et al. (1974). In addition, there is a correction for the 20% rRNA contamination ($R_0t_{1/2}$(obsd) × 0.8). [d] The number of sequences was determined by dividing the $R_0t_{1/2}$ corrected by the $R_0t_{1/2}$ for the hybridization standard. The standard chosen was the hybrid of 15S casein mRNAs with its cDNA (number average length, 1200 nucleotides); $R_0t_{1/2}$ was 1.1 × 10^{-2}. The rate of the standard reaction was corrected for the differences in cDNA size between the experimental cDNAs and the standard (Wetmur & Davidson, 1968). The rate of the standard reaction was also corrected for the difference in length between the standard 15S casein mRNAs (2520 nucleotides) and the number-average lengths of the experimental poly(A^+) RNAs shown in Table I (Hereford & Rosbash, 1977). Statistical analysis of the hybridization data indicated that the difference in numbers of total sequences between the two tissues was not statistically significant.

In order to determine the extent to which the poly(A^+) RNA sequences are shared between the two tissues, and to determine if there is a set of tumor-specific sequences, it was necessary to perform heterologous hybridizations. The extent

of hybridization observed in this type of experiment provides a measure of the extent of overlapping RNA sequences, while the kinetics of the reaction give an estimation of the relative frequencies of the shared sequences. Inspection of the hybridization curves revealed that the extent of the reaction was >95% for both the heterologous and homologous hybridizations (Figures 2 and 3). This indicated that all of the detectable poly(A^+) RNA sequences in the tumor are also found in the midpregnant mammary gland. The heterologous hybridization curve between $cDNA_t$ and RNA_{mp} (Figure 3), when compared to the homologous curve, displayed almost identical hybridization kinetics in the abundant class and small shifts of RNA frequency in the moderately abundant and scarce classes. Thus, there appear to be quantitative rather than qualitative differences between the two RNA populations.

As expected, the reciprocal heterologous reaction $cDNA_{mp}$–RNA_t (Figure 2) revealed similar results. However, a comparison of the kinetics of the homologous and heterologous reactions showed much greater shifts in RNA frequency patterns than the previous results shown in Figure 3. The abundant classes of RNA sequences found in the midpregnant mammary gland are greatly reduced in the tumor, as indicated by the very low level of hybridization in the heterologous curve at low R_0t (10^{-2}–10^0) values. There are also shifts in the frequency of different RNAs in the moderately abundant and the scarce frequency classes. As will be shown later, the abundant milk protein cDNAs comprise from 40 to 50% of the total sequences in the cDNA prepared from the midpregnant mammary gland. This predominance of a few RNA sequences would tend to decrease the concentration of sequences in a heterologous hybridization. Changes in RNA frequencies will be magnified under these conditions, resulting in a rightward shift of the $cDNA_{mp}$–RNA_t heterologous hybridization curve as compared to the $cDNA_t$–RNA_{mp}. The 95% extent of hybridization again indicated that, by using this technique, there are no detectable qualitative differences in the poly(A^+) RNA sequences found in the tumor and the normal mammary gland. However, differences in the abundances of various RNA species were observed. Examination of the thermal stability of the hybrids on hydroxylapatite yielded an identical T_m of 85 °C for both homologous and heterologous cDNA–RNA hybrids (data not shown). This indicated that the 95% hybridization observed in the heterologous reactions was due to sequence complementarity and was not the result of annealing of mismatched sequences.

Hybridization of $cDNA_t$ and $cDNA_{mp}$ to Milk Protein mRNA Sequences. The abundant RNA class in the midpregnant mammary gland, which is greatly reduced in the DMBA-induced mammary tumors (Figures 2 and 3), was assumed to consist primarily of milk protein (casein and α-lactalbumin) mRNA sequences. In order to confirm this hypothesis, both $cDNA_{mp}$ and $cDNA_t$ were hybridized to an mRNA fraction that was enriched for the milk protein mRNA sequences. This poly(A^+) RNA fraction from 8-day lactating mammary glands was enriched for these specific sequences by affinity chromatography on oligo(dT)-cellulose, preparative sucrose gradient centrifugation, and Sepharose 4B column chromatography (Rosen et al., 1975). It was composed of 90% casein and α-lactalbumin mRNA sequences as shown by agarose–urea gel analysis. The results of these hybridization experiments are shown in Figure 4. Because this mRNA fraction was composed of four milk protein sequences (15S casein doublet, 12S casein, and 10S α-lactalbumin) and a number of other mRNA species (~10%), the hybridization occurred over several log units, and a distinct transition point

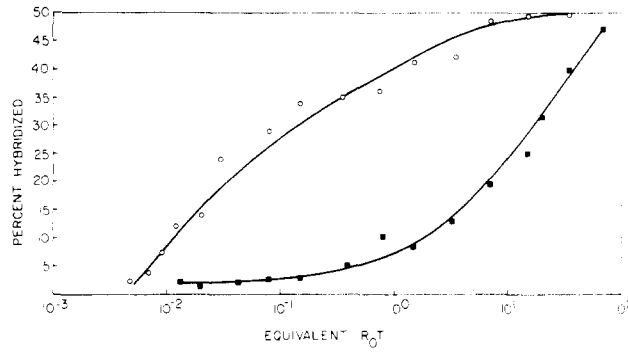

FIGURE 4: Hybridization of $cDNA_t$ and $cDNA_{mp}$ to milk protein mRNA sequences. Both $cDNA_{mp}$ (○) and $cDNA_t$ (■) were hybridized to an mRNA fraction that was specifically enriched for the milk protein sequences (casein and α-lactalbumin) at RNA concentrations ranging from 0.0017 to 0.705 mg/mL as described under Experimental Procedures. The amount of both $cDNA_{mp}$ and $cDNA_t$ was 0.12 ng.

was difficult to ascertain. However, it appears that the milk protein sequences comprise 40–50% of the total $cDNA_{mp}$ sequences, reflecting the percentage of milk protein mRNA sequences found in the poly(A^+) RNA from the midpregnant mammary gland. The curve shown in Figure 4 for the $cDNA_t$–milk protein mRNA hybridization displayed a much slower rate of hybridization. An analysis of the kinetics of hybridization suggested that the milk protein sequences were present in ~100-fold lower concentration in the tumors when compared with the normal mammary gland. This confirmed our previous results, which indicated that much lower levels of casein RNA were present in DMBA-induced mammary tumors when compared to lactating mammary glands (Rosen & Socher, 1977).

Hybridization of Poly(A^+) RNAs to [^3H]DNA. Hybridization of poly(A^+) RNAs from the DMBA-induced mammary adenocarcinomas and the normal mammary gland to heterologous cDNAs revealed no detectable qualitative differences between the two RNA populations. Because saturation hybridization is the method of choice in measuring the complexity of scarce RNA sequences, this technique was utilized in order to detect qualitative differences between the two RNA populations. RNA complexities were measured by RNA-driven hybridization to ^3H-labeled unique sequence DNA. Total cellular poly(A^+) RNAs from both the DMBA-induced mammary tumor and the normal mammary gland hybridized at saturation to 7.5% of the haploid unique sequence DNA when the annealing of the probe to any DNA contaminant in the RNA samples was subtracted (Figure 5).

The extent of hybridization suggested that both total poly(A^+) RNA populations contained a complex class of nuclear RNA sequences. In order to determine the degree to which the poly(A^+) RNA sequences were shared between the tumor and the normal gland, mixtures of the two RNAs were hybridized to the single-copy DNA probe. The mixture of tumor and midpregnant RNA saturated at 7.5%, which was the same as the two RNAs alone. If the tumor and the normal gland contained totally nonoverlapping sets of sequences, the mixture of the two would hybridize to 15%, which was the sum of the individual hybridizations. If the two tissues contain totally overlapping sets of sequences, the saturation value would be 7.5%. However, differences of less than 10–20% may not be detectable with this technique. These data also suggest that the vast majority of the poly(A^+) RNA sequences are held in common between the neoplastic and the normal tissue.

Analysis of Cell-Free Translation Products. Molecular hybridization was utilized in the present study to compare

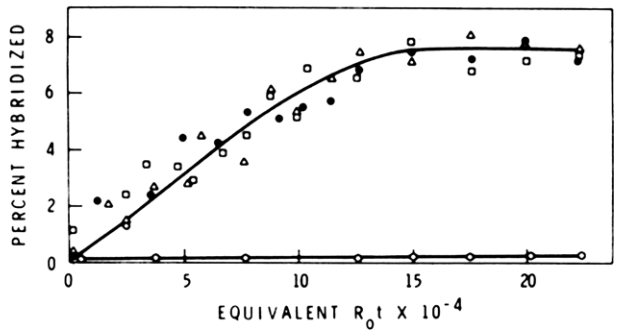

FIGURE 5: Saturation hybridization of poly(A^+) RNAs from DMBA-induced mammary tumors and 14-day midpregnant mammary glands to ^{3}H-labeled single-copy DNA. Poly(A^+) RNA from DMBA-induced mammary tumors (●) and 14-day midpregnant mammary gland (□) at RNA concentrations of 17.5 mg/mL were annealed to 0.5 ng of ^{3}H-labeled single-copy DNA. For the additivity experiment (△), the same preparations were mixed together and annealed with the same preparation of probe DNA. The concentration of tumor and midpregnant RNAs was 8.75 mg/mL. Self-annealing of the probe was determined by hybridization of the [^{3}H]DNA in the presence of oviduct RNA (○). A background zero time S_1 resistance of 2.5% was subtracted from the data before plotting. Specific activity of the probe was 4.0×10^6 cpm/μg and saturated at 85% when annealed to an excess of unlabeled rat liver DNA. The data were corrected for probe availability.

poly(A^+) RNA sequences present in the DMBA-induced mammary tumor and the normal mammary gland. An analysis of functional mRNA sequences and a comparison of the proteins coded for by these two mRNA populations were also undertaken, utilizing cell-free translation in the nuclease-treated rabbit reticulocyte system. Cell-free translation reactions were performed by using 0.5–0.75 μg of purified poly(A^+) RNAs from both the neoplastic and normal tissue, because these concentrations fell in the linear range of a translation dose–response curve.

In order to analyze the large number of translation products, it was necessary to optimize separation of the polypeptides by two-dimensional gel electrophoresis, which resolves proteins, first by isoelectric point and then by molecular weight. Equal counts per minute of [^{35}S]methionine-labeled translation products were loaded onto each isoelectric focusing gel. In order to minimize the loss of proteins, the isoelectric focusing gel was placed on the second-dimension acrylamide–NaDodSO$_4$ gel without equilibration, as described by O'Farrell (1975). One sample consisting of translation products specified by the enriched milk protein mRNA fraction (Figure 4) was included to determine the locations of the in vitro synthesized caseins and α-lactalbumin. In order to determine what polypeptides were synthesized due to endogenous mRNAs, a reticulocyte lysate sample containing no exogenous RNA was also analyzed (data not shown).

The fluorographs of the two-dimensional gels are shown in Figure 6. The predominant products synthesized in response to the RNA_{mp} were the four milk proteins. These milk proteins were also observed in the two-dimensional analysis of the tumor RNA translation products, but at much diminished levels. Further inspection of the preflashed fluorographs indicated that many of the same polypeptides were present in both the tumor and the midpregnant poly(A^+) RNA translation products. However, differences in intensities were observed that may reflect quantitative differences in specific mRNA concentrations. There are also polypeptides which appeared to be unique to either the tumor or the normal gland. This may represent either a qualitative or quantitative difference in the levels of these proteins. These experiments represent a subjective analysis of some of the proteins coded for by the two mRNA populations. Not only were there significant changes in the milk protein levels that reflected the concen-

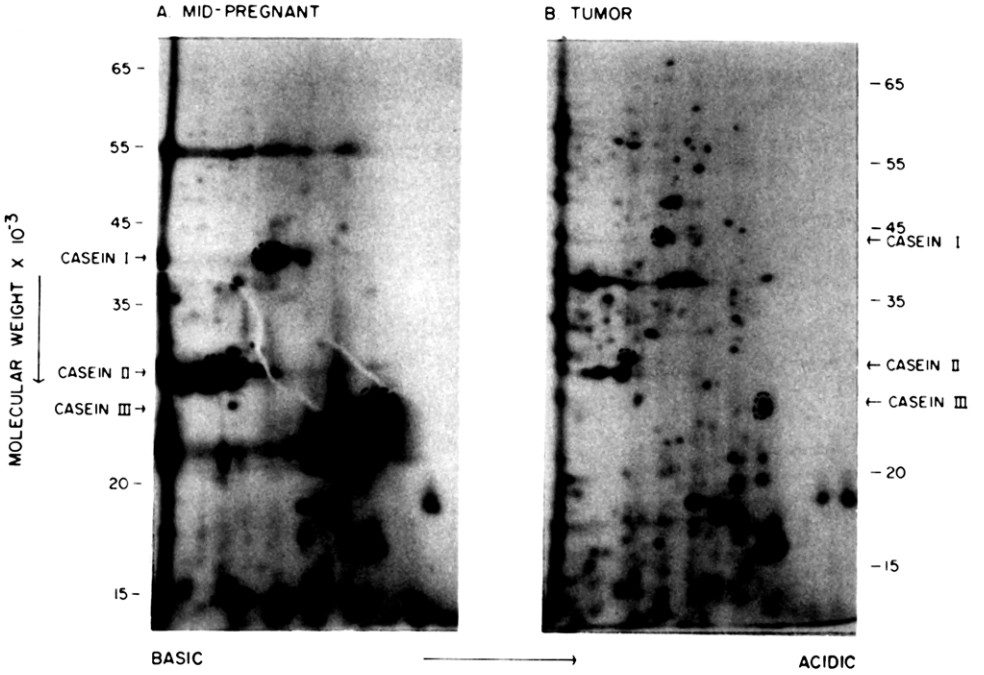

FIGURE 6: Two-dimensional analyses of cell-free translation products. Fluorograph of [^{35}S]methionine-labeled cell-free products synthesized in the nuclease-treated rabbit reticulocyte lysate system and analyzed by two-dimensional gel electrophoresis. Equal counts (500 000 cpm) were loaded onto each 4% acrylamide isoelectric focusing gel, and the second dimension was a 10% acrylamide–NaDodSO$_4$ lab gel. The gels were exposed to preflashed X-ray film for 7 days. Panel A is the fluorography of the translation products specified by the $mRNA_{mp}$, while panel B is the fluorograph of the translation products specified by the $mRNA_t$. The relative migrations of the in vitro synthesized milk proteins (casein and α-lactalbumin) in this gel system were determined by two-dimensional gel analysis of translation products from the milk protein mRNA fraction discussed in Figure 4. Translation products due to endogenous mRNA activity in the lysate system were determined by two-dimensional gel analysis of a translation assay in which no exogenous RNA was added. The three caseins in panels A and B are designated I, II, and III.

trations of the milk protein mRNA sequences but also there were numerous other quantitative and possibly qualitative differences observed between the proteins synthesized by using these two RNA populations.

Discussion

Molecular hybridization and cell-free translation have been employed by using total cellular poly(A^+) RNA populations from DMBA-induced mammary tumors and 14-day midpregnant glands in order to determine if there is a detectable set of tumor-specific RNA sequences. Total cellular poly(A^+) RNA was utilized because of the difficulties in isolating sufficient quantities of undegraded polysomal poly(A^+) RNA from these tissues (due to high nuclease activity in the DMBA-induced mammary tumors) and difficulties in isolating undegraded polysomes from the midpregnant rat mammary gland (Rosen et al., 1978). A direct comparison of nuclear and polysomal RNA populations requires careful controls to rule out cross-contamination. Furthermore, the techniques required to isolate nuclei and polysomes containing intact RNA from these tissues would have necessitated a comparison between different tissue samples. Total cellular poly(A^+) RNA, therefore, was employed in these studies. It is composed of both cytoplasmic poly(A^+) RNAs, which comprise the majority of the mass, and nuclear poly(A^+) RNAs, which contribute most of the RNA complexity.

With these reservations in mind, we employed the technique of cDNA hybridization to compare total poly(A^+) RNAs from DMBA-induced mammary tumors and normal midpregnant mammary gland. Hybridization of both poly(A^+) RNA populations to their homologous cDNAs appeared to indicate a greater complexity of tumor RNA as compared to the midpregnant RNA population. However, statistical evaluation of the hybridization data using an analysis of variance revealed that the differences in sequence complexity observed between the tumor and normal tissue (15 000 vs. 9600 sequences, respectively) were not significant.

A more accurate estimation of the relative number of sequences that are held in common between the neoplastic and normal tissues was obtained by heterologous hybridization reactions. In our experiments, both heterologous hybridization reactions annealed to >95%, which was the same extent of hybridization reached by the homologous reactions. This indicates that both RNA populations are composed largely of the same set of sequences. However, this technique is limited by the sensitivity of detecting perhaps small changes in the thousands of different scarce poly(A^+) RNA sequences. Assuming that a change of less than 2.5% (Towle et al., 1979) could not be detected, a change of 300–400 different gene products in the scarce classes, 10–20 gene products in the moderately abundant class, and 1 gene product in the abundant class would not be apparent. With this reservation, we conclude that there does not appear to be a major class of tumor-specific sequences. These data are in agreement with results obtained by similar studies between normal and transformed cells (Kuo et al., 1976; Getz et al., 1977; Williams et al., 1977) and in Novikoff hepatoma ascites cells when compared to normal liver (Reiners & Busch, 1980).

The heterologous annealing reaction was useful not only in comparing the degree of sequence homology between the two RNA preparations but also in providing information about the relative frequency distribution of different RNA sequences. Analysis of the heterologous reactions revealed that there are frequency differences between two RNA populations. The abundant RNA class from the normal mammary gland appeared to be significantly reduced in the tumor. Specifically, the milk protein RNA sequences accounted for 40–50% of the total poly(A^+) RNA from the midpregnant gland and were reduced approximately 100-fold in the tumor. This reduction of abundant, tissue-specific mRNAs has also been reported in other types of tumors (Holtzer et al., 1979; Tse et al., 1978; Reiners & Busch, 1980).

Saturation hybridizations using ^{3}H-labeled single-copy DNA were employed in order to obtain a more accurate quantitation of the high-complexity, scarce RNA sequences extracted from the neoplastic and normal tissue. Both total cellular poly(A^+) RNA populations saturated at 7.5% of the haploid single-copy genome. Since total cellular poly(A^+) RNA was used in the experiment, it appeared that much of the hybridization was due to a highly complex class of nuclear RNAs. The saturation value of 7.5% of the single-copy haploid genome is in agreement with values reported for nuclear RNAs from various rat tissues (Chikaraishi et al., 1978). In order to estimate the degree to which sequences are shared between the two tissues, mixtures of both RNAs were annealed simultaneously to the unique sequence DNA. The mixture of RNA from the tumor and the normal mammary gland hybridized to the same extent (7.5%) as either RNA alone, again indicating that the great majority of the RNA sequences is shared between the two tissues. However, these additivity experiments have a limited sensitivity. Because of the error inherent in the technique if the sequences are only partially overlapping, differences less than 10–20% may be difficult to assess. These results are not surprising in light of studies that have demonstrated that the great majority of nuclear RNAs are held in common between different tissues in the rat (Chikaraishi et al., 1978) and between different developmental stages in the sea urchin (Wold et al., 1978).

A more accurate method of detecting qualitative differences between two total RNA populations would involve isolating unique sequence DNA from RNA–DNA hybrids and then reacting this probe with homologous and heterologous RNAs as described by Ernst et al. (1979) and Grady et al. (1979). In addition, a complex class of poly(A^-) RNAs has been shown to be present in various animal tissues (Milcarek et al., 1974; Kaufmann et al., 1977; Chikaraishi, 1979). Both recycling and saturation hybridization experiments using poly(A^-) RNA populations are currently in progress in our laboratory and may reveal more subtle differences between these RNA populations.

The quantitative changes observed in the poly(A^+) RNA sequences may be reflected in the levels of protein products coded for by the two RNA populations. While hybridization studies permit an analysis of both scarce and abundant nuclear and cytoplasmic RNAs, translation experiments provide information primarily about the abundant and moderately abundant mature mRNAs, which are primarily cytoplasmic. In our studies, two-dimensional gel analyses of the translation products specified by the tumor and midpregnant mRNAs revealed a large number of newly synthesized proteins. There appeared to be quantitative differences between the two sets of translation products, which we assume reflect quantitative differences in the concentration of specific mRNA sequences or their translation efficiencies. The most striking difference was seen in the levels of the milk proteins. These proteins were present at a much lower level in the tumor when compared with the midpregnant gland. This was not surprising because of the low levels of casein mRNA detected in the tumor by molecular hybridization. There were also unique proteins that were detected on one gel but not the other. However, a large change in the concentration of a specific mRNA between the tumor and the normal gland could result in the inability to

detect a newly synthesized protein when the relative level of this specific mRNA was greatly reduced. It, therefore, would appear that a qualitative change has occurred in this type of experiment. Furthermore, because of the pH range employed during isoelectric focusing, many basic proteins could not be resolved.

Both the translation and hybridization data suggest that differences in relative abundancies of specific poly(A^+) RNAs and not the absolute presence or absence of the RNA may be important in determining which RNAs are expressed. 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 two tissues. If this is the case, then both abundant and infrequent mRNAs are important in determining the phenotype of differentiated cells. 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). While no qualitative differences were detected by the molecular hybridization experiments comparing the total poly(A^+) RNAs from the tumor and the normal mammary gland, we cannot rule out the possibility that qualitative differences exist between polysomal mRNAs between the two tissues. Large differences have been shown to exist between the sets of single copy sequences found in the polysomes of sea urchin embryos and adult tissues. Specific populations of mRNAs appear to be present on polysomes at each embyronic state (Galau et al., 1976; Hough-Evans et al., 1977), even though several investigators have concluded that nuclear RNAs of several sea urchin embryo stages and adult tissues had approximately the same sequence complexities and that their sets of nuclear RNAs are largely overlapping (Kleene & Humphreys, 1977; Hough et al., 1975; Wold et al., 1978).

The understanding of the regulation of gene expression may require an investigation of both the transcription and processing of specific mRNAs. Differences between normal and transformed mammary cells may reflect a coordinated change in both the rates of synthesis and processing of specific transcripts. Recently, we have demonstrated that the prolactin-induced accumulation of casein in the mammary gland required a 2- to 4-fold increase in the rate of transcription coupled with a 17- to 25-fold increase in the half-life of casein mRNA (Guyette et al., 1979). Thus, regulation of the relative abundancies of certain mRNA sequences, rather than an "all or none" expression, may provide one mechanism by which the levels of specific proteins and ultimately cellular functions are modulated. A careful comparison of polysomal mRNA populations is required in order to determine if selective RNA processing plays a role in the expression of the transformed phenotype in the DMBA-induced mammary carcinoma.

Acknowledgments

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Internal Motions in Deoxyribonucleic Acid II†

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ABSTRACT: We have measured the ^{1}H, ^{31}P, and ^{13}C NMR parameters of monodisperse deoxyribonucleic acid (DNA) fragments 260 and 140 base pairs long as a function of temperature and solvent viscosity. On the basis of these measurements, we calculate that within a rodlike DNA helix, the base planes, the deoxyribose sugar, and the sugar–phosphate backbone all experience large fast internal motions. The best fit of the data to a two-state model for internal motion specifies for the internal motions the following amplitudes A and correlation times τ: for the base planes, $A = \pm 20°$ and $\tau = 1 \times 10^{-9}$ s; for position 2′ of the deoxyribose sugar, $A = \pm 20$ to $\pm 33°$ and $\tau = 1 \times 10^{-9}$ s; for the P–H vectors of the sugar–phosphate backbone, $A = \pm 27°$ and $\tau = 2.2 \times 10^{-9}$ s.

In a recent study we measured the ^{31}P and ^{1}H NMR[1] parameters of short, monodisperse deoxyribonucleic acid (DNA) fragments. On the basis of that work, we were able to show that, in solution, a B DNA helix is not rigid but instead experiences fast fluctuations in the geometry of the deoxyribose sugar and of the phosphate–sugar backbone (Hogan & Jardetzky, 1979).

At that time, we modeled internal motion as being a fast two-state motion of H–H and P–H vectors relative to the long helix axis. Overall motion of the helix was treated as being that of a rod. In the context of that model, we calculated that in DNA the vector between adjacent methylene protons at deoxyribose position 2′ moves from its average geometry by ±35° with a time constant near 10^{-9} s. The three P–H vectors of the backbone phosphate were calculated to move by ±20° with a time constant also near 10^{-9} s.

We proposed that these motions could arise from a fluctuation of deoxyribose sugar pucker geometry, giving rise to a coupling of sugar and sugar–phosphate backbone motions. In small nucleotides, the conformations of the base and of the deoxyribose sugar are strongly coupled (Sundaralingam, 1975). Therefore, sugar-pucker fluctuations might be coupled to motions of the base planes in a B helix. Although narrow aromatic proton resonances could be measured in our previous study, indicating that they might be experiencing rapid internal motions, the complexity of aromatic proton relaxation made the unambiguous determination of that motion impossible.

Here, we present a detailed study of the effects of field strength, length, viscosity, and temperature upon the ^{31}P and ^{1}H relaxation properties of monodisperse fragments of B DNA. We show that the two-state model for internal motion in a rod fits well under all these experimental conditions and that under these conditions ^{31}P and ^{1}H relaxation is dominated by a simple dipolar relaxation mechanism. On the basis of these data, we calculate with greater accuracy the amplitude and time constant for internal motions in the helix. From the temperature dependence of these rates, we show that the activation energy for both phosphate and $H_{2'}$ proton motions is small, as expected for a true internal motion.

We also present, for the first time, ^{13}C NMR spectra and relaxation measurements made on a 260 base pair long DNA fragment. From measured T_1, NOE, and line-width values we show that deoxyribose sugar motions monitored by ^{13}C NMR are identical, within modeling accuracy, to the motions monitored by ^{1}H relaxation. We also show that, in addition to deoxyribose sugar motion, B DNA experiences a fast internal motion of base planes inside the helix which occurs with a time constant near 1×10^{-9} s.

Recently, Early and co-workers have obtained reasonably narrow, nearly length independent ^{1}H NMR spectra of the exchangeable aromatic H-bonded protons in long DNA helices (Early & Kearns, 1979). We have confirmed the observation with a monodisperse DNA fragment but show that proton dipolar relaxation contributes less to the line width of these protons than they have measured. Although amplitudes and time constants cannot be calculated from line widths alone, we propose that the small proton contribution to dipolar broadening of H-bonded resonances must be due to the large,

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[1] Abbreviations used: DNA, deoxyribonucleic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect.

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