Iqbal, M., Nagaraj, R., & Balaram, P. (1981) *Int. J. Pept. Protein Res.* (in press).
Irmscher, G., Bovermann, G., Boheim, G., & Jung, G. (1978) *Biochim. Biophys. Acta 507*, 470–484.
Jung, G., Konig, W. A., Liebfritz, D., Ooka, T., Janko, J., & Boheim, G. (1976) *Biochim. Biophys. Acta 433*, 164–181.
Mathew, M. K., Nagaraj, R., & Balaram, P. (1981) *Biochem. Biophys. Res. Commun. 98*, 548–555.
Mueller, P., & Rudin, D. O. (1968) *Nature (London) 217*, 713–719.
Nagaraj, R., & Balaram, P. (1981a) *Biochemistry 20*, 2828–2835.
Nagaraj, R., & Balaram, P. (1981b) *Tetrahedron 37*, 1263–1270.
Nagaraj, R., Shamala, N., & Balaram, P. (1979) *J. Am. Chem. Soc. 101*, 16–20.
Nagaraj, R., Mathew, M. K., & Balaram, P. (1980) *FEBS Lett. 121*, 365–368.
Pandey, R. C., Carter Cook, J., Jr., & Rinehart, K. L., Jr. (1977a) *J. Am. Chem. Soc. 99*, 8469–8483.
Pandey, R. C., Carter Cook, J., Jr., & Rinehart, K. L., Jr. (1977b) *J. Am. Chem. Soc. 99*, 5205–5206.
Pandey, R. C., Meng, H., Carter Cook, J., Jr., & Rinehart, K. L., Jr. (1977c) *J. Am. Chem. Soc. 99*, 5203–5205.
Prasad, B. V. V., & Balaram, P. (1981) *Int. J. Biol. Macromol.* (in press).
Prasad, B. V. V., Shamala, N., Nagaraj, R., Chandrasekaran, R. C., & Balaram, P. (1979) *Biopolymers 18*, 1635–1646.
Rao, Ch. P., Nagaraj, R., Rao, C. N. R., & Balaram, P. (1979) *FEBS Lett. 100*, 244–248.
Rao, Ch. P., Nagaraj, R., Rao, C. N. R., & Balaram, P. (1980) *Biochemistry 19*, 425–431.
Smith, G. D., Pletnev, V. Z., Duax, W. L., Balasubramanian, T. M., Bosshard, H. E., Czerwinski, E. W., Kendrick, N. E., Mathews, F. S., & Marshall, G. R. (1981) *J. Am. Chem. Soc. 103*, 1493–1501.
Stevens, E. S., Sugawara, N., Bonora, G. M., & Toniolo, C. (1980) *J. Am. Chem. Soc. 102*, 7048–7050.
Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672–676.
Veatch, W. R., Fossel, E. T., & Blout, E. R. (1974) *Biochemistry 13*, 5249–5256.
Venkatachalapathi, Y. V., & Balaram, P. (1981a) *Biopolymers 20*, 625–628.
Venkatachalapathi, Y. V., & Balaram, P. (1981b) *Biopolymers 20*, 1137–1145.
Venkatachalapathi, Y. V., Nair, C. M. K., Vijayan, M., & Balaram, P. (1981) *Biopolymers 20*, 1123–1136.
Weinstein, S., Wallace, B. A., Blout, E. R., Morrow, J. S., & Veatch, W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4230–4234.
Wuthrich, K. (1976) *NMR in Biological Research: Peptides and Proteins*, North-Holland Publishing Co., Amsterdam.
Zanacchi, R. M., & Moore, W. J. (1980) *Aust. J. Chem. 33*, 1505–1510.

Characterization of Rat Liver Oligonucleosomes Enriched in Transcriptionally Active Genes: Evidence for Altered Base Composition and a Shortened Nucleosome Repeat†

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ABSTRACT: A transcriptionally active chromatin fraction of oligonucleosome size has been separated and isolated by a modified micrococcal nuclease fractionation procedure. After mild enzymatic digestion, rat liver nuclei were lysed, and the chromatin was separated by centrifugation on linear sucrose gradients. Fractions from four regions of the gradient were pooled and labeled, from the top to the bottom, A, B, C, and D, respectively. Fraction A, which contained 20% or less of the total DNA, was determined to have a mean size of a hexanucleosome. By hybridization with [^{3}H]cDNA transcribed from total cytoplasmic poly(A) mRNA, DNA from fraction A was shown to be 10–15-fold enriched in transcribing genes when compared with total DNA. This fraction also has a somewhat higher concentration of AT base sequences. Significant differences were observed in nucleosome phasing. Fraction A has the shortest repeat length, fractions B and C are intermediate, and fraction D, which is depleted in transcribing DNA sequences, has the longest. Thus, we have isolated a chromatin fraction of oligonucleosome size enriched in transcribing genes and organized with reduced nucleosome spacing.

In differentiated cells, only a small percentage of the information in chromosomal DNA is expressed (McCarthy et al., 1973). While this transcribed chromatin appears to possess a periodic nucleosome structure (Lacy & Axel, 1975; Mathis & Gorovsky, 1976; Bellard et al., 1978), there is increasing evidence that transcribing genes exist in an altered conformation. First, transcribing nuclear DNA is preferentially digested by nucleases such as DNase I (Berkowitz & Doty, 1975; Weintraub & Groudine, 1976; Garel & Axel, 1976) and DNase II (Gottesfeld et al., 1974). In addition, micrococcal nuclease cleaves the ovalbumin gene more rapidly than the globin gene in hen oviduct (Bellard et al., 1978), and both DNase I and micrococcal nuclease preferentially cleave ac-

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tivated heat-shock genes in *Drosophila* (Wu et al., 1979). Second, certain nonhistone proteins, such as high mobility group proteins (HMG)[1] 14 and 17, have been shown to be specifically associated with transcribing chromatin and involved in maintaining the conformation which renders this DNA sensitive to DNAse I (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980). Third, nucleosome phasing has been related to transcriptional activity in several cell populations. A shorter repeat length has been correlated with an increased amount of RNA synthesis (Morris, 1976; Thomas & Thompson, 1977; Weintraub, 1978; Lohr et al., 1977a). In addition, transcriptionally active nuclei have nucleosome spacings of more variable length compared to transcriptionally inactive chromatin (Lohr et al., 1977a,b). One possible explanation of this phenomenon is that nucleosomal populations exist within a nucleus which differ in their phasing and their transcribing ability.

In order to study the structure of actively transcribing chromatin, it is desirable to isolate it in its native state. Micrococcal nuclease has been used to study the structure of transcriptionally active mononucleosomes. Levy W. & Dixon (1978) isolated trout testis nucleosome monomers which are 7-fold enriched in transcribed genes, and Bloom & Anderson (1978) isolated 5–6-fold enriched mononucleosomes from hen oviduct nuclei. By use of this methodology, transcribing mononucleosomes have been analyzed with respect to core histone complement and HMG composition (Levy W. et al., 1979; Goodwin et al., 1979; Albanese & Weintraub, 1980). By use of even lower enzyme concentrations to obtain extremely mild cleavage with micrococcal nuclease, an oligonucleosome-sized fraction of chromatin containing the majority of the nuclear RNA polymerase II has been isolated (Tata & Baker, 1978). This fraction has been found to be 5–10-fold enriched in a specific transcribed gene and is highly sensitive to DNase I (Dimitriadis & Tata, 1980). Since we were unable to achieve consistently reproducible results by using this methodology, we devised another procedure for isolating transcriptionally active chromatin in subunits larger than mononucleosome size. Using our procedure, we can isolate a 10–15-fold transcriptionally enriched fraction of DNA whose mean size, as measured by gel electrophoresis, is a hexanucleosome. This paper describes our isolation procedure and provides a partial characterization of the chromatin fractions obtained from rat liver nuclei.

Materials and Methods

Digestion of Nuclei. Liver nuclei from 11-month-old male Sprague Dawley rats (Charles River Laboratories) were purified by the method of Blobel & Potter (1966) with one alteration. A second nuclear wash was included by using 0.34 M sucrose, 50 mM Tris, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 0.2% Triton X-100. After centrifugation through a sucrose shelf, the purified nuclei were stored in 70% glycerol, 2.5 mM KH_2PO_4, pH 7, and 5 mM $MgCl_2$ at −70 °C. Prior to use, nuclei were washed twice with RSB (10 mM Tris, pH 7.4, 10 mM NaCl, and 3 mM $MgCl_2$), resuspended in RSB + 0.5 mM $CaCl_2$ at 200 A_{260} units/mL, and digested by using a modification of the procedure described by Levy W. & Dixon (1978). Micrococcal nuclease (Worthington) was added at 25 units/mL, and the nuclei were incubated on ice for 6 min with occasional shaking. This mild digestion yields less than 1% acid-soluble nucleotides. The reaction was terminated by adding EDTA to a final concentration of 2 mM. The nuclei were pelleted by centrifugation at 3000*g* for 5 min, gently resuspended in 2 volumes of 1 mM EDTA, pH 7.5, and allowed to lyse for 30 min at 4 °C.

Fractionation of Chromatin. The lysed nuclei were layered on 7–27% linear sucrose gradients (w/v) in 1 mM EDTA, pH 7.5, at a concentration of approximately 40 A_{260} units per 40-mL gradient. Care must be taken not to overload the gradients. The gradients were centrifuged in an SW27 rotor for 18 h at 26000 rpm (125000*g*) and 3-mL fractions collected by upward density displacement with an ISCO gradient collecting pump. The absorbance at 260 nm was monitored. Fractions from the top, middle, and bottom of the gradient were pooled (see Results) for further studies.

Agarose Gel and Alkaline Composite Gel Electrophoresis. Chromatin from the pooled gradient fractions was digested with Pronase (Weintraub & Groudine, 1976), and the resultant DNA was analyzed on 17.5-cm 1% agarose horizontal slab gels with Tris–acetate buffer (Loening, 1967). Electrophoresis was carried out for 24 h at 35 V. Bacteriophage λ DNA *Hin*dIII fragments and φX174 RF DNA *Hae*III fragments (Bethesda Research Laboratories Inc.) were used as markers. The gels were stained with ethidium bromide at 0.5 μg/mL and photographed on Polaroid type 55 film, under UV light, with a red filter. Prior to the hybridization studies, the length of single-stranded DNA from each fraction was measured as follows: Electrophoresis was performed on 11-cm composite agarose–polyacrylamide (0.5%:2.5%) vertical slab gels according to the method of Peacock & Dingman (1968) with the alkaline buffer system described by McDonell et al. (1977).

DNA Purification for Hybridization Reactions. Pooled chromatin fractions were precipitated in 0.1 M NaCl with 2 volumes of ethanol, and the DNA was isolated according to the method of Weintraub & Groudine (1976) with the following modifications. The Pronase digestion buffer contained 0.5% $NaDodSO_4$, and NaCl was added to 0.01 M before extraction with phenol–chloroform. After extraction, the samples were precipitated, dried, and resuspended in 10 mM Tris–5 mM EDTA, pH 7.5. Large molecular weight DNA was sonicated for 3 min with 15-s cooling intervals to yield DNA with an average single-stranded length of 400 bases, as measured by alkaline gel electrophoresis (see above). RNase A was added to a concentration of 25 μg/mL, and the samples were incubated at 37 °C for 3 h. A second Pronase digestion was carried out overnight followed by reextraction with organic solvents. The ethanol-precipitated DNA was resuspended in 5 mM EDTA and dialyzed against water. After lyophilization, the DNA was dissolved in 3 mM EDTA, pH 7, at a concentration of approximately 7 mg/mL.

Synthesis of cDNA. Polysomes were isolated from 11-month-old rat liver (Lee & Brawerman, 1971), and the polysomal RNA was extracted (Aviv & Leder, 1972). Polyadenylated mRNA was isolated by binding the RNA to an oligo(dT)–cellulose column (type 7, P-L Biochemicals) (Lasky et al., 1978). The bound polyadenylated mRNA was eluted with 10 mM Tris, pH 7.5, and 0.5% $NaDodSO_4$ and used in the synthesis of cDNA as follows. RNA (10 μg) was heated at 80 °C for 3 min in 0.1 mM EDTA and added to 50 μL of the following reaction mixture: 50 mM Tris, pH 8.3, 10 mM $MgCl_2$, 30 mM mercaptoethanol, 70 mM KCl, 0.5 mM each

[1] Abbreviations used: HMG, high mobility group proteins; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; C_0t, product of the initial DNA concentration in moles of nucleotide per liter and the time of incubation in seconds; $C_0t_{1/2}$, C_0t value at 50% hybridization; T_m, temperature at which 50% of the DNA is thermally denatured; TTP, thymidine 5′-triphosphate.

of the deoxynucleotide triphosphate, 10–40 μCi of [^{3}H]TTP, and 2.7–5 μg of oligo(dT)$_{12-18}$ (Collaborative Research, Inc.). AMV reverse transcriptase was added, and the reaction was incubated at 42 °C for 2 h. After incubation, the sample was heated to 70 °C for 3 min in 20 mM EDTA, rapidly cooled to 4 °C, adjusted to 10% glycerol, and loaded on a 2-mL Sephadex G-100 column equilibrated with TEN (10 mM Tris, pH 7.5, 1 mM EDTA, and 10 mM NaCl). The void peak was incubated in 0.3 N NaOH at 65 °C for 45 min to degrade the RNA. The remaining DNA was then neutralized, adjusted to 10% sodium acetate, pH 5, and precipitated, in the presence of 62.5 μg of tRNA/mL, with 2 volumes of ethanol. The cDNA was resuspended in TEN and stored at −20 °C.

DNA-Driven Hybridizations. Purified DNA was boiled for 15 min in 3 mM EDTA and quick-cooled to prevent reannealing. The samples were adjusted to a final concentration of 0.6 M NaCl, 20 mM Tris, pH 7.5, and 3 mM EDTA at approximately 5 mg of DNA/mL. They were diluted with hybridization buffer to obtain low C_0t values. [^{3}H]cDNA, with a specific activity of over 4×10^3 cpm/ng, was added at a ratio of 1:40 (v/v) to the sample DNA. Aliquots of 25 μL containing 1000 cpm were incubated at 66 °C for increasing time intervals. The extent of hybridization was analyzed by S1 nuclease digestion (Levy-Wilson & Dixon, 1979). After samples were flushed into digestion buffer, aliquots of 100 μL were taken for a separate estimate of the total counts per sample. To the remaining sample were added 2000 units of S1 nuclease (Miles). All samples were incubated for 1 h at 45 °C, chilled on ice, and precipitated for 15 min with 20% Cl_3CCOOH and 200 μg/mL denatured salmon sperm DNA as carrier, before collecting on filters for counting.

A cDNA self-annealing average value of 4% was subtracted from the percent hybridization of all samples. Differences in average molecular weight between the samples were corrected by multiplying the C_0t values by the square root of the ratio of 400 bases to the length of each sample (Wetmur & Davidson, 1968). C_0t values were corrected to the standard cation concentration of 0.12 M Na^+ by multiplying the values obtained by 5 (Sullivan et al., 1973).

Thermal Denaturation of DNA. Purified DNA was extensively dialyzed against 0.1 × SSC (0.015 M NaCl–1.5 mM sodium citrate, pH 7.0) prior to denaturation in this buffer. Meltings were performed in stoppered cuvettes in a Gilford 250 spectrophotometer with an electronic temperature programmer set at an increase of 0.5 °C/min. The absorbance at 260 nm was recorded against a reference buffer every 25 s. The initial DNA absorbance values were approximately 0.500. Derivative melting curves were plotted as described by Li & Bonner (1971) and Weischet et al. (1978).

Results

Fractionation of Chromatin. In order to devise a procedure for the isolation of transcriptionally active chromatin which results in a consistent yield of oligonucleosomal-sized fragments, mild micrococcal nuclease digestion conditions were developed (see Materials and Methods). Conditions used are based on the method of Levy W. & Dixon (1978) with several significant modifications. The Ca^{2+} concentration was lowered to 0.5 mM, and the nuclease digestion was carried out at 4 °C for 6 min. Following digestion, the nuclei were lysed and the chromosomal fragments separated on a sucrose gradient (Figure 1). It can be seen that this fractionation scheme produces oligonucleosome-sized chromatin containing only a small mononucleosome component. The digestion conditions employed in our procedure correspond to a release of approximately 2% of the nucleic acid into the 3000*g* supernatant

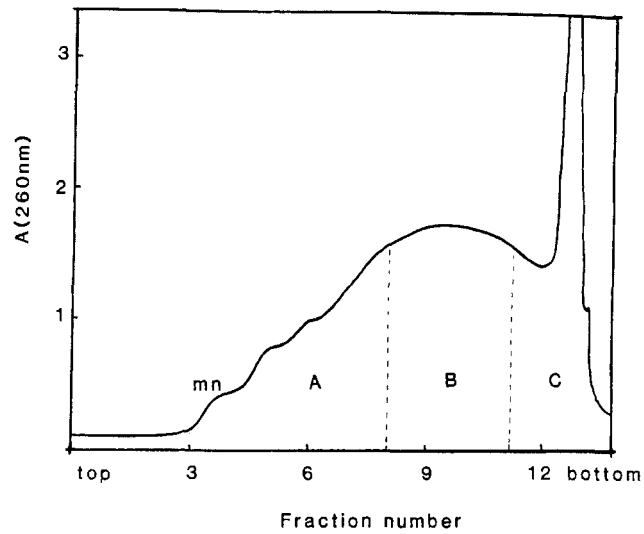

FIGURE 1: Sucrose-gradient profile of a typical fractionation experiment. Sedimentation is from left to right. Fraction A contains 19% of the original chromatin, fraction B contains 39% of the material, and fraction C contains 20%. The pellet, fraction D, also contains 20% of the chromatin. The mononucleosome peak is labeled mn. Fractions (3 mL) were collected, and the samples were pooled as indicated by the dotted lines. Fractions 1–3 were discarded.

Table I: Size Distribution of DNA from the Chromatin Fractions

	fraction A	fraction B	fraction C	fraction D
lowest detectable band				
base pairs	~264	393	368	425
multimer no.	1	2	2	2
highest detectable band				
base pairs	2579	5598	8843	>24000
multimer no.	14	28	46	>112
mean weight size				
base pairs	1087	2426	3580	~3134
multimer no.	6	12	18	~15

and to less than 1% of the A_{260} of the sample rendered acid soluble. The fractions collected from the sucrose gradient were combined as shown in Figure 1. The initial fractions with a constantly increasing slope of absorbance and consisting of 20% or less of the chromatin were labeled fraction A. The broad peak of chromatin from the middle of the gradient was labeled fraction B, and the sharp peak at the bottom of the gradient was labeled fraction C. Approximately 20% of the chromatin remained at the bottom of the centrifuge tube. This material was collected and labeled fraction D. The distribution of nucleic acids in fractions A, B, C, and D displayed in Figure 1 is 19%, 39%, 20%, and 20%, respectively.

Range and Mean Size of DNA. Analysis by agarose gel electrophoresis of the DNA extracted from the pooled fractions shows that the chromatin in each fraction is comprised of multiples of approximately 200 base pairs. Table I shows the range and mean size of the DNA from each fraction. There is a progressive increase in mean DNA size from fraction A, a hexanucleosome, to fraction C. Fraction D, on the other hand, contains a much wider range of DNA fragments than any of the other fractions and a mean DNA size intermediate between that found for fraction B and fraction C.

Hybridization Reactions. In order to evaluate our cDNA probe, DNA excess hybridization reactions were performed between total rat DNA and [^{3}H]cDNA copied from total rat liver cytoplasmic polyadenylated mRNA. The hybridization curve for the association of cDNA with total rat DNA is shown in Figure 2A along with a reannealing curve for total rat DNA (Holmes & Bonner, 1974). The hybridization of cDNA to

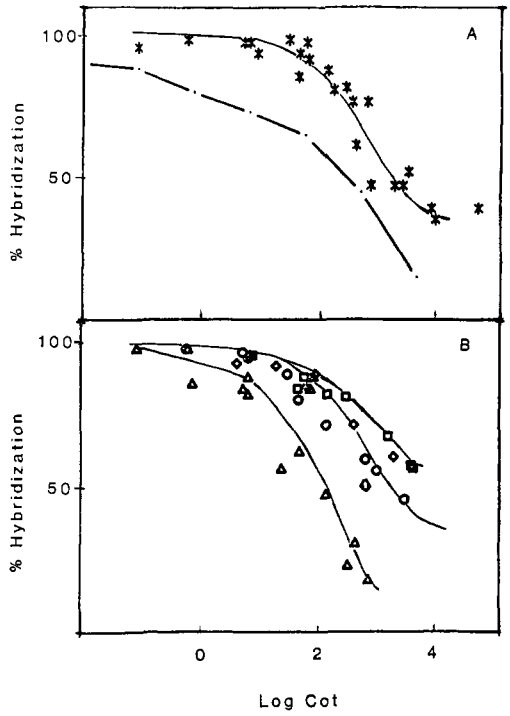

FIGURE 2: Hybridization of [^{3}H]cDNA, complementary to rat liver poly(A) mRNA, with rat DNA. Materials were obtained and reactions performed as described in the text. (A) Reaction of cDNA with total rat DNA (*); reannealing of rat DNA (— · —). (B) Reaction of cDNA with fraction A DNA (△); reaction of cDNA with fraction B DNA (◇); reaction of cDNA with fraction C DNA (○); reaction of cDNA with fraction D DNA (□). This figure incorporates the data from three separate experiments. The hybridization curve of cDNA to total DNA shown in Figure 2A is redrawn in Figure 2B for comparative purposes. The hybridization data from fractions B and C fall on this curve.

Table II: Comparison of Hybridization Kinetics of DNA from the Chromatin Fractions

chromatin sample	app $C_0t_{1/2}$	enrichment ($C_0t_{1/2}$ unfractionated/ $C_0t_{1/2}$ sample)
fraction A	2.25×10^2	11.0
fraction B	4.47×10^3	0.6
fraction C	1.58×10^3	1.6
fraction D	$>10^4$	0.2
unfractionated	2.51×10^3	1.0

total rat DNA occurs over C_0t values more representative of unique copy sequences than repetitive sequences. In addition, the entire reaction occurs over 2 orders of magnitude, showing second-order kinetics and indicating the presence of only one major sequence complexity class. Thus, our cDNA appears to represent only unique copy genes.

The hybridization kinetics of this cDNA probe to total DNA and DNA from the chromatin fractions are shown in Figure 2B. Fraction A hybridizes to the cDNA with significantly faster kinetics than any of the other samples. Approximate $C_0t_{1/2}$ values of all the fractions are listed in Table II. In this experiment, fraction A is 11-fold enriched in mRNA sequences. Fractions B and C are similar to unfractionated DNA, and fraction D is extremely depleted in transcribing sequences with an extrapolated $C_0t_{1/2}$ value $>10^4$. The slight enrichment of transcribing sequences in fraction C may reflect the presence of active genes which are particularly resistant to micrococcal nuclease such as those which have a high GC composition (Roberts et al., 1962).

Thermal Denaturation of DNA. An analysis of the relative base composition of each fraction was made by using thermal

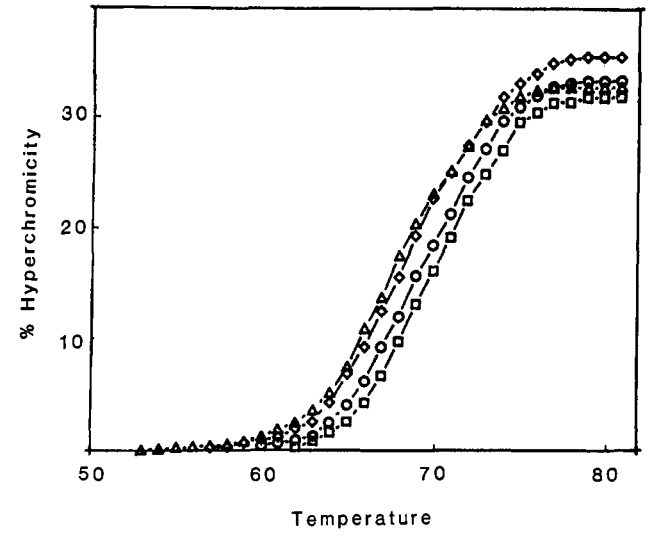

FIGURE 3: Thermal denaturation curves of DNA purified from the chromatin fractions. (△) Fraction A DNA (T_m 67.5 °C); (◇) fraction B DNA (T_m 68.5 °C); (○) fraction C DNA (T_m 69.5 °C); (□) fraction D DNA (T_m 70 °C). The denaturation buffer is 0.1 × SSC. The percent hyperchromicity is approximately the same for each sample, and the relatively sharp transition reflects the absence of any significant amount of single-stranded DNA. Thermal denaturation experiments were performed twice on each of two separately fractionated and purified sets of DNA samples. The data obtained from each of these experiments were basically identical. Figure 3 represents one of these four experiments.

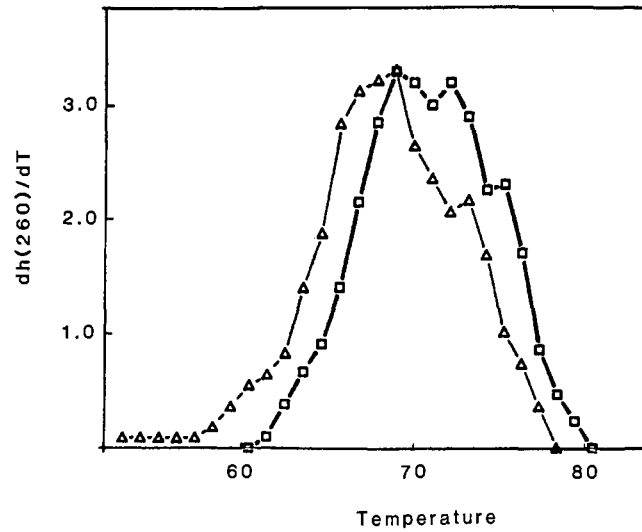

FIGURE 4: Derivative melting profiles of DNA from fractions A (△) and D (□). Low-melting transition I occurs between 56 and 61 °C, transition II between 62 and 70 °C, and high-melting transition III between 71 and 79 °C. The area under each transition for the DNA from all four fractions is shown in Table III.

denaturation studies. DNA purified from the chromatin fractions melted in sharp transitions as shown in Figure 3. The T_m values obtained from these curves increase slightly from 67.5 °C for fraction A to 70 °C for fraction D. These thermal melting transitions can be plotted as derivative melting curves to permit further analysis of the compositions. Figure 4 contains the derivative melting curves for fraction A and fraction D DNA. The melting curves for fractions B and C are not shown in this figure, but a comparison of the data from the melting transitions for all four fractions is given in Table III. Three transitions are observed, similar to the three thermal denaturation components for rat DNA resolved by McConaughy & McCarthy (1970). A minor melting transition between 57 and 62 °C makes up 7%, 2%, and 1% of the

Table III: Percent Area under Each Transition of Derivative Melting Profiles[a]

chromatin sample	transition I, 56–61 °C (%)	transition II, 62–70 °C (%)	transition III, 71–79 °C (%)
fraction A	7	65	29
fraction B	4	60	36
fraction C	2	54	44
fraction D	1	51	48

[a] Derivatives were calculated over 1 °C intervals from thermal denaturation curves, and the percent area for each transition was determined by adding the derivative values within each temperature range.

Table IV: Nucleosome Repeat Length of Fractionated and Unfractionated Rat Liver Chromatin

source of chromatin	repeat length (base pairs)[a]
fraction A	183
fraction B	202
fraction C	195
fraction D	219
unfractionated	193, 190,[b] 197[c]

[a] The average size of the DNA in each band was determined by using the mobilities of *Hin*dIII λDNA and *Hae*III φX174 DNA fragments as standards. The nucleosome repeat length was calculated from the slope of a plot of fragment size higher than a tetramer (Compton et al., 1976) vs. fragment number. Data were accumulated from four independent experiments; the final repeat length values were an average of these results. [b] Calculated from the data of Thomas & Thompson (1977). [c] Calculated from the data of Compton et al. (1976).

hyperchromicity of fractions A, C, and D, respectively. This transition, which reflects the denaturation of highly AT-rich sequences, is thus enriched in fraction A and depleted in fractions C and D.

The second transition of the derivative melting curve occurs between 62 and 71 °C and reflects the melting of moderately AT-rich sequences. This transition is the major melting component in fractions A and B while the denaturation of the GC-rich sequences, transition III, makes up a relatively small component of these two fractions. On the other hand, transition III is enriched in fractions C and D compared to fractions A and B.

Sizing of the Nucleosome Repeat. The nucleosome repeat length of each fraction was measured by electrophoresis of purified DNA on horizontal agarose gels. A calibration curve constructed from the simultaneous migration of *Hin*dIII λ DNA and *Hae*III φX174 DNA fragments was used to size the nucleosome multimers from the pentamer to the highest limit of resolution. We were able to clearly resolve nucleosome multimers up to decamers or dodecamers as shown in Figure 5. The DNA repeat length of each sample was determined from the slope of a plot of fragment size vs. fragment number. This method of calculating the repeat length from the average size difference between successive fragments eliminates the effect of exonucleolytic degradation on DNA length (Kornberg, 1977). The size of the nucleosome repeat for each chromatin fraction is listed in Table IV. Fraction A has the shortest repeat of 183 base pairs, B and C have similar intermediate values of 202 and 195 base pairs, respectively, and D has the longest repeat of 219 base pairs. It is clear that the extent of digestion does not correlate with the repeat length since fraction B has a shorter mean length than fraction C but the same or a slightly longer repeat length. On the other hand, excellent correlation exists between a shorter nucleosome repeat length and an enrichment in transcribed genes.

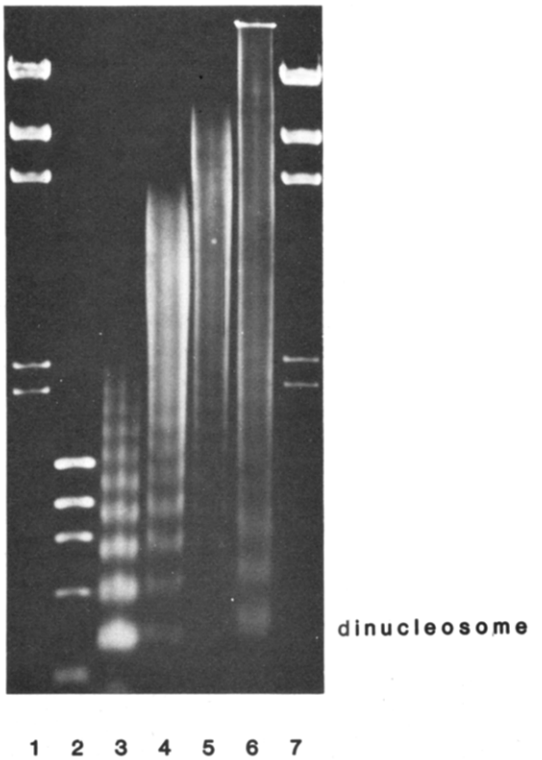

FIGURE 5: Agarose gel electrophoresis of DNA purified from the chromatin fractions by Pronase digestion (see Materials and Methods). Migration distances of nucleosomal DNA were determined, and nucleosome repeat lengths were calculated as described under Results. Lanes 1 and 7 contain *Hin*dIII digested λ DNA; lane 2 contains *Hae*III digested φX174 DNA; lane 3 contains fraction A DNA; lane 4 contains fraction B DNA; lane 5 contains fraction C DNA; lane 6 contains fraction D DNA.

Discussion

We have described a technique for the isolation from rat liver of a chromatin fraction of oligonucleosome size which is enriched in transcribing genes. This method was based on previous studies demonstrating the susceptibility of active chromatin to nuclease digestion. However, these experiments, using moderate digestion conditions [75 units of enzyme/mL, 1.5 min, 0.5 mM $CaCl_2$, 37 °C (Bloom & Anderson, 1978), or 25 units of enzyme/mL, 20 min, 1 mM $CaCl_2$, 37 °C (Levy W. & Dixon, 1978)], produced active chromatin of mononucleosome or core particle size. Tata & Baker, (1978), using extremely mild digestion conditions (0.1–0.2 unit of enzyme/mL, 1.5 min, 0.25 mM $CaCl_2$, 25 or 29 °C), were able to isolate an oligonucleosome-sized chromatin fraction containing high levels of RNA polymerase II. Since we wished to study the conformation of oligomeric transcribing chromatin and since we were unable to achieve reproducible DNA cleavage by using the mild digestion procedure of Tata and Baker, we devised an alternative fractionation and isolation technique (25 units of enzyme/mL, 6 min, 0.5 mM $CaCl_2$, 4 °C). This protocol produced an extremely consistent low level of DNA cleavage such that in each reaction three criteria were achieved, namely, (a) less than 1% of the A_{260} was rendered acid soluble, (b) less than 2% of the A_{260} was released from the nuclei during digestion, and (c) the median size distribution of nucleosome DNA from fraction A was approximately a hexanucleosome with very little monomer DNA present. Occasionally enzyme preparations were used which had a lower than average nuclease activity when assayed at 4 °C. When these enzymes were used, the $CaCl_2$ concen-

tration was elevated slightly such that the three digestion criteria were satisfied.

Since liver is made up of a heterogeneous population of cells, it is possible that, in our fractionation procedure, we have selected for the genome of a single transcriptionally active cell type rather than the transcriptionally active chromatin from all the cells, but we consider this possibility rather unlikely. First, in a mixed population of nuclei, micrococcal nuclease shows relatively little specificity for one nuclear type over another, although the rates of digestion may vary somewhat (E. M. Berkowitz and A. Sanborn, unpublished observation). Second, under the very limited digestion conditions used, it is extremely improbable that a nuclear population would be cleaved completely to yield chromatin fragments between mononucleosomal and dodecanucleosomal size as is found in our transcriptionally active fraction.

The association of rat DNA with [^{3}H]cDNA copied from total rat liver cytoplasmic polyadenylated mRNA has a $C_0t_{1/2}$ value of approximately 2.5×10^3 and is similar to data from previously reported experiments (Gottesfeld, 1978). The cDNA hybridization with this DNA and the DNA from fractions A, B, and C (see Figure 2) all go essentially to completion and vary only in the $C_0t_{1/2}$ values and therefore in the concentration of sequences complementary to polyadenylated liver mRNA. However, fraction D DNA shows only limited hybridization to the cDNA, indicating a major loss in complementary sequences. The differences in $C_0t_{1/2}$ values between fraction C and total DNA (less than 2-fold) are very small, but the increased hybridization does correspond to a small decrease in the nucleosome repeat length. Studies are now being carried out to ascertain if these differences are significant. On the other hand, the increase in hybridization kinetics with fraction A and the decrease with fraction D are highly significant and relate to substantial changes in repeat length.

The T_m of the DNA from each fraction showed a small progressive increase in going from fraction A to fraction D. This change in T_m may in part reflect an increase in mean size, but the observed difference of 2–3 °C is greater than the predicted effect of size alone which would be approximately 0.5 °C (Crothers et al., 1965). Therefore, the major factor responsible for the melting change is probably an increase in the GC base composition between the fractions (Marmur & Doty, 1962). Fraction C is slightly enriched in transcribed genes and contains a larger mean size of DNA fragments (Table I) than fractions A and B. This suggests that a small number of transcribed sequences are GC rich and are partially protected from micrococcal nuclease digestion. Giri & Gorovsky (1980) have shown that this is the case for ribosomal genes in *Tetrahymena*. On the other hand, fraction A is highly enriched in transcribing genes and contains a higher percentage of AT base sequences. It is possible, therefore, that a substantial amount of transcribing DNA is AT rich.

One approach used in studying the organization of basic chromatin structure is an analysis of the nucleosome repeat length. Several studies have compared nucleosome phasing in nuclei which vary in transcriptional activity and found that actively transcribing nuclei contain shorter repeat lengths. These studies include chicken liver vs. erythrocytes (Morris, 1976), rabbit cerebral cortical neurons vs. cerebral cortical neuroglia, cerebellar neurons, and liver (Thomas & Thompson, 1977), sea urchin gastrula vs. sperm (Spadafora et al., 1976), HeLa and yeast vs. chicken erythrocytes (Lohr et al., 1977a) and developing chicken embryo erythrocytes (Weintraub, 1978). All these experiments measured an average nucleosome repeat length for each nuclear population. However, it would seem likely, since only a small percentage of the genome is transcribing, that a decreased mean repeat length might not solely reflect transcriptional ability. On the other hand, evidence indicates that, even within a single cell type, the phasing of nucleosomes is heterogeneous (Lohr et al., 1977a,b; Johnson et al., 1976; Martin et al., 1977). It is possible that heterogeneous nucleosome phasing is due to the presence of several chromosomal populations within a nucleus, each varying in repeat length and transcriptional activity. In fact, our results support this interpretation. Fraction A, which is >10-fold enriched in transcribing sequences, contains the shortest nucleosome repeat length of 183 base pairs, and fraction D, which is depleted in transcribing sequences, has the longest repeat length of 219 base pairs. Fractions B and C, which have hybridization kinetics similar to those of total chromatin, have intermediate repeat lengths of 202 and 195 base pairs, respectively. Therefore, these experiments are the first direct demonstration of the existence of classes of nucleosomes within a nuclear population which show a correlation between short repeat length and increased transcriptional activity.

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References

Albanese, I., & Weintraub, H. (1980) *Nucleic Acids Res. 8*, 2787–2805.

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408–1412.

Bellard, M., Gannon, F., & Chambon, P. (1978) *Cold Spring Harbor Symp. Quant. Biol. 42*, 779–791.

Berkowitz, E. M., & Doty, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3328–3332.

Blobel, G., & Potter, V. R. (1966) *Science (Washington, D.C.) 154*, 1662–1665.

Bloom, K. S., & Anderson, J. N. (1978) *Cell (Cambridge, Mass.) 15*, 141–150.

Compton, J. L., Bellard, M., & Chambon, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4382–4386.

Crothers, D. M., Kallenbach, N. R., & Zimm, B. H. (1965) *J. Mol. Biol. 11*, 802–820.

Dimitriadis, G. J., & Tata, J. R. (1980) *Biochem. J. 187*, 467–477.

Garel, A., & Axel, R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3966–3970.

Giri, C. P., & Gorovsky, M. A. (1980) *Nucleic Acids Res. 8*, 197–214.

Goodwin, G. H., Mathew, C. G. P., Wright, C. A., Venkov, C. D., & Johns, E. W. (1979) *Nucleic Acids Res. 7*, 1815–1835.

Gottesfeld, J. M. (1978) *Philos. Trans. R. Soc. London, Ser. B 283*, 343–357.

Gottesfeld, J. M., Garrard, W. T., Bagi, G., Wilson, R. F., & Bonner, J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2193–2197.

Holmes, D. S., & Bonner, J. (1974) *Biochemistry 13*, 841–848.

Johnson, E. M., Littau, V. C., Allfrey, V. G., Bradbury, E. M., & Matthews, H. R. (1976) *Nucleic Acids Res. 3*, 3313–3329.

Kornberg, R. D. (1977) *Annu. Rev. Biochem. 46*, 931–954.

Lacy, E., & Axel, R. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3978–3982.
Lasky, L., Nozick, N. D., & Tobin, A. J. (1978) *Dev. Biol. 67*, 23–39.
Lee, S. Y., & Brawerman, G. (1971) *Biochemistry 10*, 510–516.
Levy, W., B., & Dixon, G. H. (1978) *Nucleic Acids Res. 5*, 4155–4163.
Levy W., B., Conner, W., & Dixon, G. H. (1979) *J. Biol. Chem. 254*, 609–620.
Levy-Wilson, B., & Dixon, G. H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1682–1686.
Li, H., & Bonner, J. (1971) *Biochemistry 10*, 1461–1470.
Loening, U. E. (1967) *Biochem. J. 102*, 251–257.
Lohr, D., Corden, J., Tatchell, K., Kovacic, R. T., & Van Holde, K. E. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 79–83.
Lohr, D., Tatchell, K., & Van Holde, K. E. (1977b) *Cell (Cambridge, Mass.) 12*, 829–836.
Marmur, J., & Doty, P. (1962) *J. Mol. Biol. 5*, 109–118.
Martin, D. Z., Todd, R. D., Lang, D., Pei, P. N., & Garrard, W. T. (1977) *J. Biol. Chem. 252*, 8269–8277.
Mathis, D. J., & Gorovsky, M. A. (1976) *Biochemistry 15*, 750–755.
McCarthy, B. J., Nishiura, J. T., Doenecke, D., Nasser, D. S., & Johnson, C. B. (1973) *Cold Spring Harbor* Symp. *Quant. Biol. 38*, 763–771.
McConaughy, B. L., & McCarthy, B. J. (1970) *Biochem. Genet. 4*, 425–446.
McDonell, M. W., Simon, M. N., & Studier, F. W. (1977) *J. Mol. Biol. 110*, 119–146.
Morris, N. R. (1976) *Cell (Cambridge, Mass.) 9*, 627–632.
Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668–674.
Roberts, W. K., Dekker, C. A., Rushizky, G. W., & Knight, C. A. (1962) *Biochim. Biophys. Acta 55*, 664–673.
Spadafora, C., Bellard, M., Compton, J. L., & Chambon, P. (1976) *FEBS Lett. 69*, 281–285.
Sullivan, D., Palacios, R., Stavnezar, J., Taylor, J. M., Faras, A. J., Kiely, M. L., Summers, N. M., Bishop, M. M., & Schimke, R. T. (1973) *J. Biol. Chem. 248*, 7530–7539.
Tata, J. R., & Baker, B. (1978) *J. Mol. Biol. 118*, 249–272.
Thomas, J. O., & Thompson, R. J. (1977) *Cell (Cambridge, Mass.) 10*, 633–640.
Weintraub, H. (1978) *Nucleic Acids Res. 5*, 1179–1188.
Weintraub, H., & Groudine, M. (1976) *Science (Washington, D.C.) 193*, 848–856.
Weisbrod, S., & Weintraub, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 631–635.
Weisbrod, S., Groudine, M., & Weintraub, H. (1980) *Cell (Cambridge, Mass.) 19*, 289–301.
Weischet, W. O., Tatchell, K., Van Holde, K. E., & Klump, H. (1978) *Nucleic Acids Res. 5*, 139–160.
Wetmur, J. G., & Davidson, N. (1968) *J. Mol. Biol. 31*, 349–370.
Wu, C. Wong, Y., & Elgin, S. C. R. (1979) *Cell (Cambridge, Mass.) 16*, 807–814.

Effect of Tautomeric Shift on Mutation: N^4-Methoxycytidine Forms Hydrogen Bonds with Adenosine in Polymers†

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ABSTRACT: N^4-Methoxycytidine (mo^4C), previously found to act only as uridine (U) in transcription [Singer, B., & Spengler, S. (1981) *Biochemistry 20*, 1127], was tested for its ability to base pair as U in copolymers of (U,mo^4C) annealed with poly(A) or transcribed with ATP and DNA-dependent RNA polymerase. Mixing curves have now indicated that the derivative is retained in a poly(U,39% mo^4C)·poly(A) helix, unlike unmodified C in poly(U,35% C). The presence of 13–39% mo^4C in U polymers lowered the melting temperature, T_m, observed in annealed complexes both with poly(A) and after transcription with ATP. However, complexes isolated after transcription had a large hyperchromicity and melted cooperatively, which indicated that they are hydrogen bonded. The decreased T_m for poly(U,mo^4C)·poly(A) compared to that for poly(U)·poly(A) can be attributed to stacking changes and adjacent base-pair disruption by mo^4C. The greater cooperative melting of transcribed poly(U,39% mo^4C) as compared to the annealed complex may indicate that the methoxy substituent is normally a mixture of rotamers and that the syn rotamer is required for transcription. The interference of the methoxy substituent was also shown by the loss of helix formation by poly(C,mo^4C) in acid solution. mo^4C decreased the T_m much more than A, which stacks well in acid. U, which neither stacks nor participates in an acid structure, caused more distortion than either of the other bases. It is inferred that mo^4C has the base-pairing ability of U but that the planarity of the substituent is lost.

Previous studies in this laboratory on the effects of modified nucleotides on transcription of polymers by DNA-dependent RNA polymerase [reviewed by Singer (1981)] have shown that modifications which block essential hydrogen-bonding sites produce high levels of ambiguity (Kröger & Singer, 1979a). Modifications on exocyclic groups not necessarily blocking these sites cause a variety of effects (Singer & Spengler, 1981). N^4-Methoxycytidine (mo^4C)[1] appears to be only in the imino

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[1] Abbreviations used: mo^4C, N^4-methoxycytidine; ho^4C, N^4-hydroxycytidine; m^6A, N^6-methyladenosine; i^6A, N^6-isopentenyladenosine; m^4C, N^4-methylcytidine; ϵA, 1,N^6-ethenoadenosine; ϵC, 1,N^4-ethenocytidine; SSC, 0.15 M NaCl–0.015 M sodium citrate; N indicates any nucleotide in a polynucleotide while the single letters A, U, and C are used for the base moieties in a polynucleotide; HPLC, high-pressure liquid chromatography.

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