Distribution of Deoxyribonucleic Acid Sequences in Fractionated Chromatin*

Jacob D. Duerksen† and Brian J. McCarthy‡

ABSTRACT: The possibility of fractionating chromatin into active and inactive portions was investigated by controlled shearing and differential centrifugation. The effectiveness of this fractionation of chromatin from normal or malignant mouse cells is suggested by the distribution of satellite DNA sequences and several distinguishable melting components between the pellet and supernatant fractions. The more condensed fraction of sheared chromatin is richer in satellite sequences, whereas the extended fraction is deficient. The various chromatin fractions differ also in their thermal denaturation profiles, suggesting that portions of the genome other than the repetitive satellite sequences are separable by this method.

Similar studies were carried out with chromatin isolated from testicular cells of the crab, *Cancer magister*. The most readily sedimentable fractions are enriched in both the poly(d(A-T)) satellite and the (G-C)-rich satellite. These findings extend the generalization that highly redundant satellite DNA sequences tend to be specifically localized in the most condensed regions of interphase chromosomes.

Several recently published reports suggest that a considerable degree of transcriptional fidelity is retained when purified chromatin is used to synthesize RNA *in vitro* (Paul and Gilmour, 1966, 1968, 1969; Huang and Huang, 1969; Bekhor *et al.*, 1969; Smith *et al.*, 1969). Thus, the populations of RNA molecules synthesized on chromatin isolated from different tissues are readily distinguishable; but each chromatin preparation produces RNA very similar to that synthesized *in vivo* in the tissue from which it originates. These results, although admittedly crude and preliminary, indicate that the controlling elements which influence selective transcription largely reside in the structure of the interphase chromosomes themselves. This conclusion implies that advances in our understanding of the regulation of gene expression in eucaryotes might result from a detailed study of chromatin structure. In particular, the possibility arises of fractionating chromatin into active and inactive portions.

In fact, few attempts at chromatin fractionation have been reported since the separation of apparently active and inactive portions of thymus chromatin by Frenster *et al.* (1963). However, more recently Yunis and coworkers have reported the specific localization of satellite DNA in the most condensed portions of both mouse and guinea pig chromatin (Yasmineh and Yunis, 1969; Yunis and Yasmineh, 1970). In both cases these satellite DNAs are highly repetitive in base sequence as well as distinctive in base composition (Waring and Britten, 1966; Flamm *et al.*, 1969; Britten and Kohne, 1968; Kit, 1961). In this paper we present data which confirm the conclusions of Yasmineh and Yunis (1969) for mouse chromatin and also demonstrate that fractions of mouse chromatin differ in other respects.

In order to permit an extension of these findings to other kinds of animals, we have attempted to fractionate chromatin from invertebrate tissues. For this purpose, species of crab in the genus *Cancer* appear to be particularly favorable biological material. These crabs contain a satellite DNA component (Sueoka, 1961) which consists mainly of alternating d(A-T) base sequences (Schwartz *et al.*, 1962). Various species contain quite different proportions of this satellite DNA (Smith, 1963). The satellite DNA is present in cells of several tissues (Klett *et al.*, 1969; Teng and Sueoka, 1964) and appears to be located in the cell nucleus (Astell *et al.*, 1969). An earlier claim that the d(A-T) satellite is mitochondrial DNA has recently been corrected (Skinner *et al.*, 1970). More recently, a second satellite of high G-C content has also been demonstrated in the DNA of members of the same and another genus of crabs (Skinner, 1967). Thus, these cells have DNA with two easily resolvable satellite components, at least one of which is highly redundant in base sequence. Fractionation experiments show that both satellite DNAs are specifically enriched in the most highly condensed portions of testicular cell chromatin isolated from *Cancer magister*.

Materials and Methods

Isolation of Chromatin. The preparation of chromatin was based on the methods of Marushige and Bonner (1966) as described by Bonner *et al.* (1968). Various modifications were introduced as specified for the various tissues which served as starting material.

TLT hepatoma cells (Taper *et al.*, 1966) were grown in the ascites form in Swiss Webster mice. Cells were harvested and cooled rapidly by addition of equal volumes of ice-cold saline–EDTA buffer (0.075 M NaCl and 0.024 M EDTA, pH 8.0). They were then washed three times with equal volumes of the same buffer to remove the majority of the ascites fluid and blood. Washed, packed cells from four mice were homogenized in a motorized Teflon-glass homogenizer in 10 ml of cold saline–EDTA buffer. Passage through several layers of cheesecloth was followed by centrifugation at 1500*g* for 15 min. The crude nuclear pellet was washed three times by resuspension in approximately 30 ml of saline–EDTA buffer and cen-

* From the Department of Microbiology, University of Kansas School of Medicine, Kansas City, Kansas 66103 (J. D. D.) and from the Departments of Biochemistry and Genetics, University of Washington, Seattle, Washington 98105 (B. J. M.). *Received October 1, 1970.*

† Present address: Department of Biology, University of Calgary, Calgary, Alberta. This research was carried out at the University of Washington while on leave of absence and was supported by U. S. Public Health Service Research Fellowship GM-18,335 and U. S. Public Health Service Research Grant GM-12,449. Publication costs paid by the National Research Council of Canada (A-6123).

‡ To whom to address correspondence.

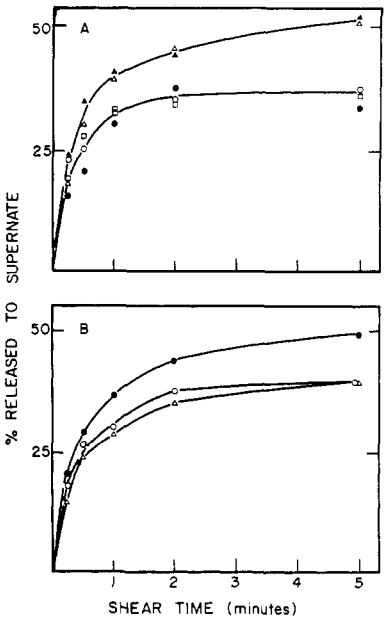

FIGURE 1: Shearing characteristics of chromatin from several mouse tissues. (A) 1.5 ml each of [^{14}C]thymidine-labeled liver chromatin at 15 (●), 30 (□), and 60 μg per ml (○) were sheared for increasing times and 0.2-ml aliquots taken after each time increment and centrifuged at 3000*g* for 15 min. Similarly, samples of ^{14}C-labeled kidney chromatin at 30 μg/ml were sheared and centrifuged on two separate occasions (△ and ▲). (B). ^{14}C-labeled chromatin at 30 μg/ml from spleen (●), brain (○), and thymus (△) were similarly sheared and centrifuged. Duplicate 50-μl samples of each supernatant were counted.

trifuged at 1500*g* for 15 min. Resuspension of the pellet in 0.05 M Tris (pH 8.0) was usually followed by two washes. The washed nuclei, resuspended in 10 ml of 0.05 M Tris buffer, were homogenized with several light strokes of the motorized Teflon-glass homogenizer. The crude chromatin was dispersed by hand homogenization (Dounce) with buffered sucrose to give a final sucrose concentration of 10%. Aliquots (10 ml) were layered on discontinuous sucrose gradients composed of a 15-ml bottom layer of autoclaved 55% sucrose buffered with 0.01 M Tris (pH 8.0) and a 10-ml top layer of 45% sucrose. The chromatin was sedimented by centrifugation in the SW27 head of the Spinco Model 2-65B for 2.5–3 hr at 25,000 rpm. The clear yellow chromatin pellet was resuspended by hand homogenization in 5 ml of 0.01 M Tris and the residual sucrose removed by dialysis against 0.01 M Tris overnight or by centrifugal washing with 0.01 M Tris and 10,000*g* for 20 min. When the spectral characteristics (Bonner *et al.*, 1968; Chalkley and Jensen, 1968) indicated appreciable contamination by extra protein, the sucrose gradient centrifugation step was repeated. The purified chromatin was diluted to give a chromatin suspension containing about 0.5 mg of DNA/ml. Aliquots were rapidly frozen in a Dry Ice and alcohol bath and stored at −90°. The preparation and destruction of cells and nuclei was routinely monitored microscopically using trypan blue stain. In cases where the DNA was labeled with radioactive thymidine, the preparation was also monitored isotopically. Material contaminating the chromatin preparation and found at the sucrose concentration interphases after centrifugation contained considerably less than 1% of the total radioactivity.

The same methods were used in this examination of chromatin of the common Dungeness crab (*C. magister*). The method outlined by Astell *et al.* (1969) was used to remove spermatocytes from testicular cells. Nuclei and chromatin were prepared from these cells by methods analogous to those employed for various mouse tissues.

Preparation of DNA. The preparation of DNA from nuclei of mouse liver (McCarthy and Hoyer, 1964) was by the method of Marmur (1961) as modified by Church and McCarthy (1967).

The isolation of the small quantities of DNA from whole and fractionated chromatin was accomplished by the CsCl method used by Bekhor *et al.* (1969); 1 ml of the chromatin preparation was diluted with 1 ml of 8 M CsCl and centrifuged for 16–20 hr at 35,000 rpm in 2-ml tubes in the Spinco 40 rotor. The protein pellicle and the CsCl solution were siphoned off and the DNA pellet dissolved in a small volume of 0.01 M Tris (pH 8.0) and dialyzed against the same buffer. Ninety-six per cent or more of the DNA from labeled chromatin preparations was found in the pellet. *Bacillus subtilis* DNA was prepared as described elsewhere (McCarthy and McConaughy, 1968).

Isotopic Labeling of DNA. TLT hepatoma cells were labeled by injecting mice intraperitoneally in the fifth day of cell growth with 200 μCi of [^{3}H]thymidine. Twenty-four hours later an additional 200 μCi was inoculated. At 40–48 hr the cells were collected for preparation of chromatin. DNA isolated from this chromatin had a specific activity of approximately 100 cpm/μg. Labeling of DNA of various mouse organs was accomplished by inoculating 18 2-day-old Swiss Webster mice intraperitoneally at 0 and 24 hr with 50 μCi of [2-^{14}C]-thymidine. At 48 hr chromatin was prepared from brain, thymus, liver, spleen, and kidney. DNA isolated from these various chromatins had specific radioactivities between 125 and 260 cpm per μg with liver, spleen, and kidney DNA being the highest.

Shearing of Chromatin. Sonication of chromatin in a Branson sonifier resulted in variable extents of shearing and degradation of DNA. Shearing of TLT hepatoma chromatin by sonication at apparent maximum power output for 10 sec or less released little material that was not sedimented by centrifugation at 10,000*g* for 15 min. However, sonication for 20–45 sec sheared the chromatin to a point where very little material was pelleted. Consequently, this method of shearing was abandoned in favor of homogenization in the Virtis 45 which proved easier to control.

A Virtis "45" homogenizer fitted with three of the smallest blades and the flanged 2-ml capacity stainless steel cup was used. A small shallow plastic jar containing ice surrounded the steel cup. This homogenizer has been used by others (Bonner *et al.*, 1968; Chalkley and Jensen, 1968; Bekhor *et al.*, 1969) for shearing chromatin. The degree of shearing varies with the type of cup, the power input, and the time. The 2-ml capacity, flanged cup coupled with a power input of 80 V was chosen for all the experiments to be described; and the time of shearing varied as specified.

Fractionation of Sheared Chromatin. Subsequent to shearing, chromatin was fractionated by simple differential centrifugation at 3000*g* for 15 min to produce a supernatant fraction and a pellet similar to the operational definition used by Frenster *et al.* (1963). A more refined fractionation was effected by centrifugation in a high concentration, steep sucrose gradient (Loeb, 1967; Dolbeare and Koenig, 1968; Chalkley and Jensen, 1968). Aliquots of sheared chromatin preparations were brought to 0.2 M sucrose concentration and layered on 14-ml or 37-ml linear sucrose gradients (0.25–2.5 M sucrose in 0.01 M Tris, pH 8.0). These tubes were centrifuged in the SW40.1 or SW27 head, respectively, on a Spinco 2-65B centri-

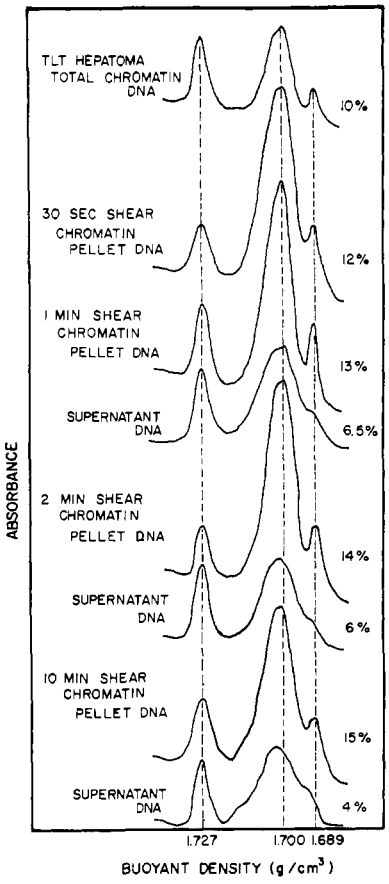

FIGURE 2: Analytical pycnography of DNA isolated from several mouse hepatoma chromatin fractions. Chromatin supernatant and pellet fractions obtained after shearing and centrifugation were analyzed by CsCl density gradient centrifugation using *Myxococcus xanthus* DNA as a reference.

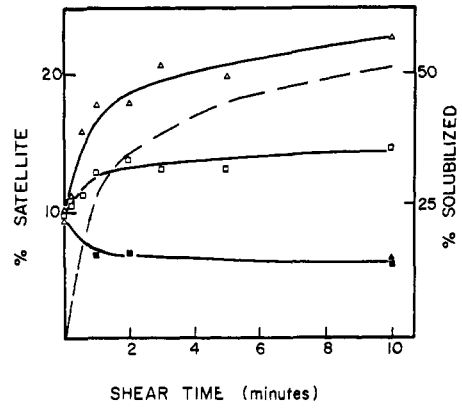

FIGURE 3: The content of mouse satellite DNA in fractionated chromatin. DNA was obtained from pellet and supernatant fractions prepared according to the procedure outlined in the legend to Figure 1. The content of mouse satellite was estimated from the melting profiles of supernatant (▲) or pellet (△) fraction DNA and from densitometer tracings of analytical pycnographic determination of pellet (□) or supernatant (■) DNA in the Model E ultracentrifuge. A curve describing the kinetics of solubilization of chromatin by shearing is included for purposes of comparison.

fuge at a speed equivalent to an average of 50,000*g* for 60 min. Fifteen- to twenty-drop fractions were collected after tube puncture.

Melting Profiles of DNA and Chromatin. Melting profiles of DNA or chromatin were carried out in either 0.01 M Tris (pH 8.0) or SSC (0.15 M NaCl–0.015 M sodium citrate) in 1-ml stoppered cuvets. A Gilford Model 2000 multiple-sample absorbance recorder was used to follow changes in absorbance during heating at a constant rate of approximately 1°/min. In each case the pH change due to increasing temperature did not exceed −0.4 unit through the melting range. To avoid serious aggregation or interference by scattering when determining the melting profile of chromatin fractions, the initial A_{260} was set between 0.150 and 0.300 with a full-scale deflection on the recorder of 0.2. The melting profiles were analyzed by the use of normal probability paper according to the method of Knittel *et al.* (1968).

Analytical Density-Gradient Centrifugation of DNA in CsCl. An aliquot containing between 1 and 3 μg of DNA was added to saturated CsCl along with 20X SSC to give a final concentration of 1X SSC and a refractive index in an Abbé-refractometer of 1.4000 (initial density of 1.7067 g/cm³). Analyses were conducted with a Spinco Model E analytical ultracentrifuge with a standard ultraviolet absorption optical system in the Spinco An F rotor. Centrifugation was conducted at 25° for 20–24 hr at 42,040 rpm. Photographic records made on Kodak commercial film were analyzed with a Joyce-Loebl densitometer. The relative amounts of main band and satellite DNA were estimated by planimetry. *Myxococcus xanthus* DNA (*d* 1.727 g/cm³) was used as a reference.

Results

Kinetics of Shearing of Mouse Chromatin. The rate of release of chromatin material into the 3000*g* supernatant is similar for the chromatin obtained from mouse brain, thymus, liver, spleen, and kidney (Figure 1). The kinetics of shearing under the conditions used are essentially independent of chromatin concentration through the range corresponding to 15–333 μg of DNA/ml. The amount solubilized levels off at a value between 35 and 50% after some 2 min of shearing. The reproducibility of the shearing procedure is evident from the correspondence between the results for the two different hepatoma preparations and the same kidney preparation sheared on different days (Figure 1).

Satellite DNA Content of Fractionated Chromatin. Representative tracings of analytical CsCl centrifugation analysis of DNA isolated from total, pellet, and supernatant fractions of mouse hepatoma chromatin are presented in Figure 2. There is an increase in the satellite content of sheared chromatin pellet DNA from 10% at zero time to approximately 15% after 5-min shearing. A decreased amount of satellite is found in the chromatin supernatant DNA. These results are summarized in Figure 3. There is a progressive enrichment for satellite in the pellet as the shear time is increased.

Thermal Denaturation Profiles of DNA Isolated from Fractions of Mouse Chromatin. The relative proportions of satellite and main components in DNA isolated from various chromatin fractions were also determined by analysis of thermal denaturation profiles. The data were transformed so that it could be plotted on normal probability paper as suggested by Knittel *et al.* (1968). This method greatly facilitates an accurate estimation of the relative proportions of two or more DNA components (McConaughy and McCarthy, 1970). These results are also summarized in Figure 3. Estimates of satellite content for chromatin pellet fractions appeared routinely higher than those determined by CsCl analytical pycnography.

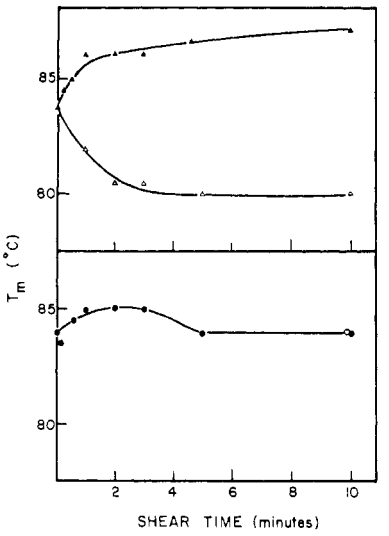

FIGURE 4: T_m of mouse hepatoma chromatin fractions (upper frame) and the DNA isolated from them (lower frame). Mean thermal denaturation temperatures were determined from melting profiles of the supernatant (△) and resuspended pellet (▲) fractions or the DNA isolated from them (○, ●). Fractions were obtained after shearing as outlined in the legend to Figure 1. Chromatin T_m's were determined in 0.01 M Tris buffer (pH 8.0) and DNA T_m's in SSC.

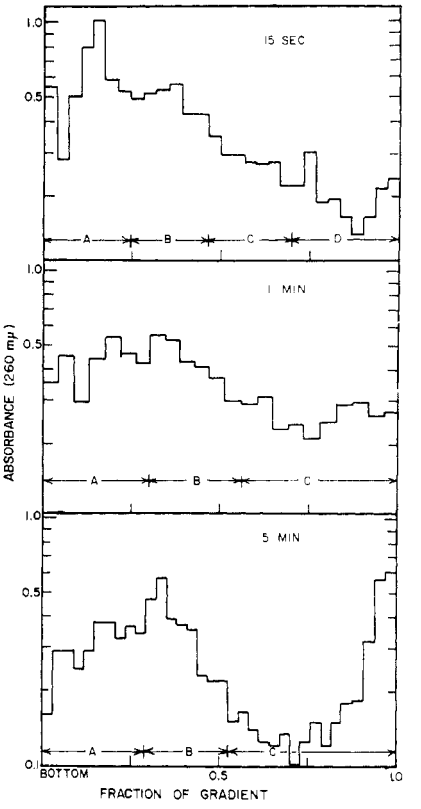

FIGURE 5: Sucrose density gradient centrifugation of sheared crab testis chromatin. Aliquots (1.5 ml) of chromatin at 530 μg/ml were sheared for the indicated times. After shearing, 1 ml of each sample was brought to 0.2 M with sucrose and layered on a 37-ml 0.25–2.5 M sucrose gradient. After centrifugation at 50,000g for 60 min in a Spinco SW27 swinging-bucket rotor, fractions were collected, diluted, and the absorbance at 260 mμ for each was determined. The combined fractions A, B, C, and D were analyzed for DNA components as described in Figure 8 and by the melting profile technique (Knittel *et al.*, 1968), as for Tables I and II.

Presumably, this effect results from the higher hyperchromicity of (A-T)-rich DNA compared to the main component (Mahler *et al.*, 1964). However, the same general pattern exists, indicating a paucity of satellite sequences in the solubilized material.

Thermal Denaturation Profiles of Fractionated Chromatin. The temperature at which chromatin reveals a hyperchromic effect is a function of the amount and kinds of protein associated with the DNA (Bonner *et al.*, 1968). The difference between the melting profiles of pellet and supernatant chromatin isolated after shearing is most readily apparent from comparison of the mean thermal denaturation temperature or T_m (Figure 4). The T_m of the pellet fractions increases as a function of shear time, while that of the supernatant fractions decreases. This effect is not ascribable to fractionation of the DNA itself according to G-C content, since the T_m's of DNA isolated from pellet or supernatant show no systematic variation (Figure 4). Together, these results suggest that parts of the genome other than the mouse satellite DNA are nonrandomly distributed among separable fractions of the interphase chromosomes.

Kinetics of Shearing of Crab Testis Chromatin. The rate of release of chromatin material into the 3000g centrifuged supernatant is similar to that for the chromatin obtained from mouse tissue (Figure 1). An examination of the profile (Figure 5) of sucrose density gradient centrifugation of chromatin samples sheared for 15 sec, 1 min, and 5 min, demonstrates a progressive change. The majority of the material in the chromatin sheared for 15 sec was found toward the bottom of the gradient. After 5-min shearing, the major broad peak appeared in the bottom half of the gradient, although a considerable proportion remained at the top of the tube. The profile for the 1-min sheared sample was intermediate. Again, as for mouse chromatin, considerable heterogeneity in sedimentation behavior is evident.

Satellite DNA Content of Fractionated Crab Chromatin. Chromatin sheared for various times was fractionated by centrifugation. DNA was isolated from both pellet and supernatant fractions and examined by analytical centrifugation. One difficulty arising from such a determination is that of estimating the relative areas under the two peaks. This problem is accentuated by shearing since the molecular weight of the DNA is progressively reduced, and the peaks become broader. However, as shown in Figure 6, an accurate determination of the relative peak areas can be made throughout a range of molecular weights. In this experiment, total crab DNA was sheared for various times in the Virtis homogenizer and the percentage of d(A-T) satellite determined. Even with the relatively broad peaks, an accurate estimate of the relative proportions of the two components appears feasible.

The tracings of the DNA analyses made on some of the crab chromatin fractions are displayed in Figure 7. In all cases, the pellet fractions are enriched in d(A-T) satellite content. This effect is most convincing for the pellet fractions isolated after larger periods of shear. Conversely, the two supernatant chromatin fractions are deficient in d(A-T) satellite content. These results also confirm the presence of a small (G-C)-rich satellite DNA (Skinner, 1967). The relative content of this minor DNA component in the various chromatin fractions appears to parallel that of the d(A-T) satellite, although the difficulty of quantitatively estimating such a minor component by planimetry makes this conclusion tentative. The enrichment in the pellet fractions for both of these satel-

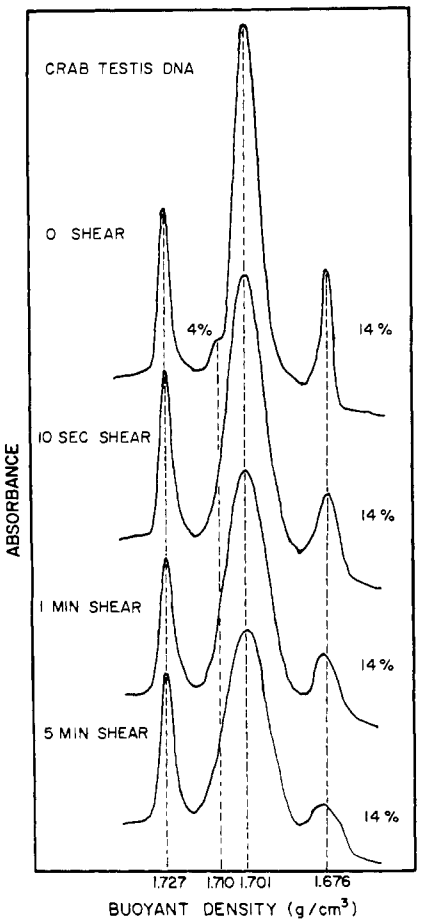

FIGURE 6: Analytical pycnography of crab testis DNA. Unsheared DNA and samples sheared for 10 sec, 1 min, and 5 min were analyzed by CsCl density gradient centrifugation using *Myxococcus xanthus* as reference DNA. The percentage figures refer to estimates of the content of the two satellite DNAs made by planimetry.

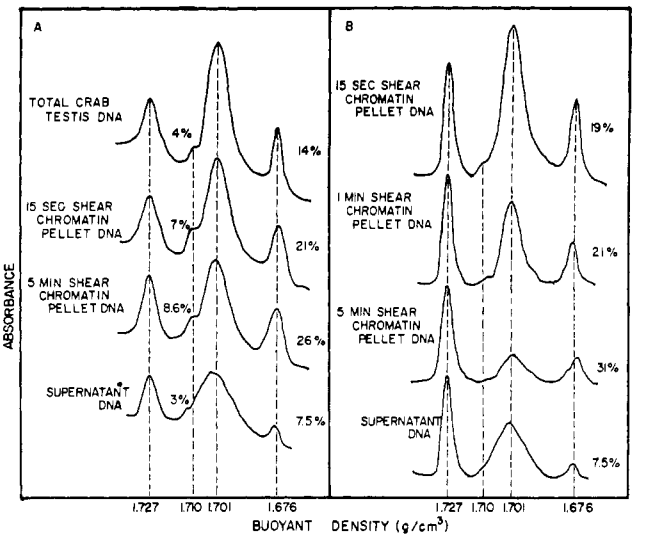

FIGURE 7: Analytical pycnography of DNA isolated from crab testis chromatin fractions. Total crab testis DNA isolated by the method of Marmur (1961), and DNA isolated by the CsCl centrifugation method from supernatant and pellet chromatin fractions after shearing for various times, were analyzed by analytical density gradient centrifugation. (A) Samples (1 ml) of crab testis chromatin at 650 μg/ml were sheared for the indicated times, each centrifuged at 3000*g* for 15 min, and DNA isolated from the resultant pellets and supernatants. (B) DNA was isolated from the pellet and the supernatant chromatin fractions obtained after various periods of shearing.

lite DNAs as a function of the time of shear is summarized in Table I.

In a similar analysis of DNA isolated from chromatin after shearing and sucrose density gradient centrifugation fractionation (Figure 5), a progression of enrichment for d(A-T) satellite was observed (Figure 8). The bottom fractions (A) from the 15-sec shear gradient exhibited the highest satellite contents (20%), whereas the lowest percentages (10%) were found in the top fractions (D). The middle fractions (B) of both the 1- and 5-min shear gradients had the highest satellite contents of 25 and 27%, respectively. The top fractions (C) of each showed low satellite contents of 7 and 10%, similar to the corresponding fractions of the 15-sec gradient. These results, as well as the satellite content determined from the melting profiles, are also summarized in Table I. The value determined from the melting profile was routinely higher than that determined by analytical centrifugation, presumably because of the higher hyperchromicity of (A-T)-rich DNA (Mahler *et al.*, 1964).

Melting Profiles of Fractionated Crab Chromatin and DNA. Melting profiles of DNA and chromatin fractions analyzed by the normal probability plot (Knittel *et al.*, 1968) present a useful method of characterization. Figure 9 shows such a plot for *B. subtilis*, a *B. subtilis–E. coli* (4:1) mixture, total crab testis DNA, and the DNA isolated from a 15-sec shear chromatin pellet. The *B. subtilis* melting curve gives rise to a straight line, whereas the *B. subtilis–E. coli* mixture reveals the resolution of the two components having different melting ranges. Total crab testis DNA denatured in SSC has four melting components: the 14% d(A-T) satellite (Smith, 1963), two main components, and a (G-C)-rich component. These same four components are represented in DNA isolated by the CsCl method from a portion of fractionated chromatin. Other fractions of chromatin contained the same four components identified by thermal denaturation profiles although their proportions differed somewhat.

When the denaturation of crab testis DNA was carried out in 0.01 M Tris with a very gradual rate of temperature increase (approximately 0.5°/min) additional structure in the profile became evident. The d(A-T) satellite region in the total DNA

TABLE I: d(A-T) Satellite Content of DNA Obtained from Pellet and Supernatant Chromatin Fractions and Various Combined Sucrose Density Gradient Chromatin Fractions.

Shear Time:	15 sec		1 min		5 min	
	% d(A-T)		% d(A-T)		% d(A-T)	
Fraction	CsCl Profile	Melt. Profile	CsCl Profile	Melt. Profile	CsCl Profile	Melt. Profile
Pellet	19.5	22	21	26	31	27
Supernatant					7.7	19
A	20	22	19.5	25	19.5	30
B	18	29	23.5	27	27	32
C	16	28	7.5	23	10	22
D	11					

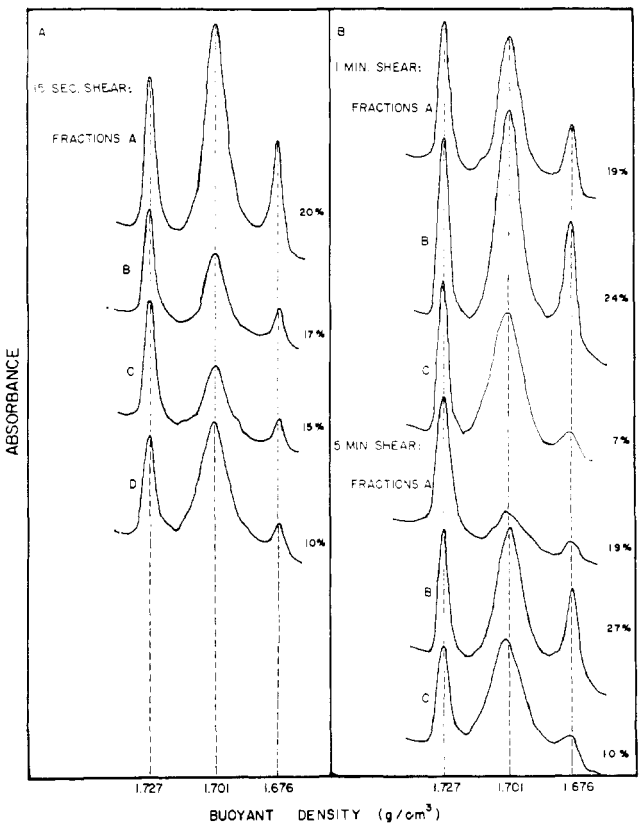

FIGURE 8: Analytical pycnography of DNA isolated from crab testis chromatin fractions. The sucrose density gradient fractions of the different sheared chromatin preparations were combined as indicated in Figure 5: and DNA was isolated from these pools A, B, C, and D.

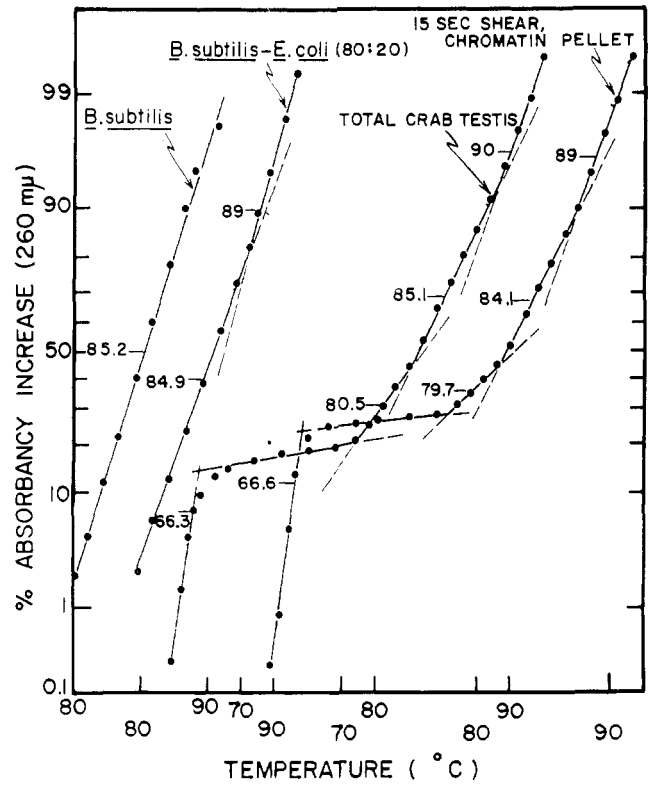

FIGURE 9: Probability plot of normalized melting profiles of microbial and crab DNA samples. Denaturation was carried out in 1 × SSC; from left to right *B. subtilis* DNA, a mixture of *B. subtilis* DNA (80%) and *E. coli* DNA (20%), total crab testis DNA, and 15-sec sheared chromatin pellet DNA.

melting profile was resolved into two components apparently differing in base composition (Brzezinski *et al.*, 1969). In addition, the T_m's of the DNA prepared from chromatin fractions by the CsCl method were several degrees higher than those for the total crab testis DNA purified by the Marmur (1961) method. This result suggests that the small amount of chromatin protein surviving the DNA extraction (Bekhor *et al.*, 1969) remains bound to the DNA and affects the melting characteristics of each component. The absence of this effect in melting curves carried out in SSC (Figure 9) may be attributable to the higher temperatures necessary to achieve denaturation, which may also lead to dissociation of the residual protein. Nevertheless, the melting profiles of DNAs obtained from supernatant, pellet, and sucrose density gradient fractions for sheared chromatin revealed the same number of components, but in quite variable proportions. Any more detailed analysis of the number of melting components requires further work. However, a comparison of the average T_m's in Table II implies a considerable difference between total, supernatant, and pellet chromatin fractions.

TABLE II: T_m of Crab Testis Chromatin Fractions and DNAs.[a]

Shear Time:	15 sec	1 min	5 min
Fraction	T_m	T_m	T_m
Chromatin			
Total	84.6	84.3	82.6
Supernatant	82.2	81.0	79.6
Pellet	87.5	85.5	84.4
DNA			
Total[b]	67.8	67.8	67.7
Supernatant			71.5
Pellet	71.5	72.2	71.2
A	69.8	68.7	68.0
B	68.8	68.0	67.8
C	68.9	67.6	67.6

[a] Denaturation was carried out in 0.01 M Tris, pH 8.0. [b] Total DNA was isolated by the procedure of Marmur (1961), while the other DNAs were isolated by the CsCl method (Bekhor *et al.*, 1969).

No systematic differences in T_m are apparent for DNA extracted from these various fractions. Entirely analogous conclusions can be drawn from the analyses of melting profiles of DNA isolated from sucrose density gradient fractions. Again, the same four components appear, although in somewhat different proportions. No differences among the three average T_m's are resolvable (Table II).

Discussion

Earlier studies published by Frenster *et al.* (1963) suggest that chromatin preparations may be sheared and separated by by centrifugation into fractions corresponding to cytologically defined euchromatin and heterochromatin. These authors attempted to demonstrate a functional separation in terms of the relative transcriptional activity on the part of the two frac-

tions. An alternative approach depends upon a demonstration of the separation of different classes of DNA base sequences.

Chromatin preparations were isolated from mouse tissues since the mouse genome contains an easily distinguishable satellite DNA which is highly redundant in base sequence (Waring and Britten, 1966) and yet distributed on a proportionate basis among chromosomes of the various size classes (Maio and Schildkraut, 1969). Controlled shearing of mouse chromatin, followed by low-speed centrifugation, results in a differential distribution of satellite DNA in the pellet and supernatant fractions. In chromatin prepared from a variety of mouse tissues, DNA segments containing satellite sequences proved to be more resistant to shear forces. Possibly this indicates preferential location of satellite sequences in more highly condensed regions of the chromatin. Such a conclusion is consistent with the proposition of Pardue and Gall (1970) that satellite DNA is associated with pericentromeric heterochromatin. More severe degradation of chromatin by sonication is apparently capable of separating chromatin fractions consisting almost entirely of satellite base sequences (Yasmineh and Yunis, 1969). Together, these observations imply that at least a portion of the repetitive DNA sequences are present in highly condensed regions, around the centromere of probably a majority of the mouse chromosomal set. This view is consistent with the observation of Flamm *et al.* (1969), indicating that mouse satellite sequences are seldom, if ever, transcribed; this suggests a structural or regulatory role.

Examination of thermal denaturation profiles of chromatin preparations suggests the presence of several distinguishable components. The melting profiles of sheared chromatin are separable into several components whose proportions differ between the supernatant and pellet fractions. This data has not been presented here in detail since its significance is not yet established. Nevertheless, even a comparison of the mean thermal denaturation temperatures shows progressive differences between supernatant and pellet fractions. This observation implies that the fractionation of mouse chromatin is not limited to the specialized location of mouse satellite DNA, but also extends to other chemically distinct chromosomal regions. Parallel experiments failed to reveal differences in the proportion of redundant and unique DNA sequences among the various chromatin fractions (McCarthy and Duerksen, 1970).

The d(A-T) satellite of crab DNA is perhaps the best example of a highly repetitive species-specific component in eucaryotic cell DNA. It is certainly the most highly repetitive, having a basic repeating sequence of only two bases (Schwartz *et al.*, 1962), compared to some 6 bases for the guinea pig α-satellite and 8–13 for the mouse satellite DNA (Southern, 1970). Moreover, in certain species it may represent a very large fraction, up to 35% of the total DNA (Smith, 1963). In all of these cases, evidence now exists which associates these three satellite DNAs with the most highly condensed portion of the interphase chromosomes. In the case of crab DNA, a second satellite DNA of high G-C content also appears to be located in such restricted regions, although no evidence is yet available as to its degree of nucleotide sequence repetition.

In each of these cases of satellite DNA, the localization in condensed heterochromatin masses implies genetic inactivity. However, convincing evidence for this exists only for mouse satellite DNA which appears to be rarely, if ever, transcribed in several kinds of cells (Flamm *et al.*, 1969). Nevertheless, from the very repetitious nature of the base sequences themselves, coding for the production of a protein appears to be most unlikely. Thus, many authors concerned with the function of these satellite DNAs have concluded that they may play a structural role in chromosome function (Pardue and Gall, 1970; Jones, 1970; Hennig and Walker, 1970). However, no detailed model delineating the role of repetitive sequences has yet appeared.

Other authors have concerned themselves with the mechanism by which such sequences are generated (Britten and Kohne, 1968; Hennig and Walker, 1970; Southern, 1970), invoking either sudden saltations or accepted classical mechanisms which involve unequal crossover. Whatever the route by which they appear in a given species, one must also consider the possible selective advantage by which they are maintained as part of the genome. We propose that their continued presence is as much a result of the lack of efficient mechanisms operating to eliminate them as of selective advantages which they confer upon the species. For even if they do play a structural role in chromosome organization, it is difficult to understand why the amount of satellite DNA is so variable among species. This is especially true for the extreme variability in the amount of d(A-T) satellite in various species of the genus *Cancer* (Smith, 1963). This same principle of the relative inefficiency of mechanisms operating to eliminate superfluous DNA from a genome may be applicable to other situations in which seemingly inactive sequences are present.

References

Astell, C. R., Suzuki, D. T., Klett, R. P., Smith, M., and Goldberg, I. H. (1969), *Exp. Cell Res. 54*, 3.

Bekhor, I., Kung, G. M., and Bonner, J. (1969), *J. Mol. Biol. 39*, 351.

Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimoto, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B., and Widholm, J. (1968), *Methods Enzymol. 12B*, 3.

Britten, R. J., and Kohne, D. E. (1968), *Science 161*, 529.

Brzezinski, A., Szafranski, P., Johnson, P. H., and Laskowski, M., Sr. (1969), *Biochemistry 8*, 1228.

Chalkley, R., and Jensen, R. H. (1968), *Biochemistry 7*, 4380.

Church, R. B., and McCarthy, B. J. (1967), *J. Mol. Biol. 23*, 459.

Dolbeare, F., and Koenig, H. (1968), *J. Cell Biol. 39*, 35a.

Flamm, W. G., Walker, P. M. B., and McCallum, M. (1969), *J. Mol. Biol. 40*, 423.

Frenster, J. H., Allfrey, V. G., and Mirsky, A. E. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 1026.

Hennig, W., and Walker, P. M. B. (1970), *Nature (London) 225*, 915.

Huang, R. C. C., and Huang, P. C. (1969), *J. Mol. Biol. 39*, 365.

Jones, K. W. (1970), *Nature (London) 225*, 912.

Josse, A., Kaiser, A. D., and Kornberg, A. (1961), *J. Biol. Chem. 236*, 864.

Kit, S. (1961), *J. Mol. Biol. 3*, 711.

Klett, R. P., Chabot, I. M., and Smith, M. (1969), *Can. J. Biochem. 47*, 829.

Knittel, M. D., Black, C. H., Sandine, W. E., and Fraser, D. K. (1968), *Can. J. Microbiol. 14*, 239.

Loeb, J. E. (1967), *Biochim. Biophys. Acta 145*, 427.

Mahler, H. R., Kline, B., and Mehrota, B. D. (1964), *J. Mol. Biol. 9*, 801.

Maio, J. J., and Schildkraut, C. L. (1969), *J. Mol. Biol. 40*, 203.

Marmur, J. (1961), *J. Mol. Biol. 3*, 208.

Marushige, K., and Bonner, J. (1966), *J. Mol. Biol. 15*, 160.

McCarthy, B. J., and Duerksen, J. D. (1970), *Cold Spring Harbor Symp.* (in press).
McCarthy, B. J., and Hoyer, B. H (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 915.
McCarthy, B. J., and McConaughy, B. L. (1968), *Biochem. Genet. 2*, 37.
McConaughy, B. L., and McCarthy, B. J. (1970), *Biochem. Genet. 4*, 425.
Pardue, M. L., and Gall, J. G. (1970), *Science 168*, 1356.
Paul, J., and Gilmour, R. (1966), *J. Mol. Biol. 15*, 242.
Paul, J., and Gilmour, R. (1968), *J. Mol. Biol. 34*, 305.
Paul, J., and Gilmour, R. (1969), *J. Mol. Biol. 40*, 137.
Schwartz, M. N., Trautner, T. A., and Kornberg, A. (1962), *J. Biol. Chem. 237*, 1961.
Skinner, D. M., Beattie, W. G., Kerr, M. S., and Graham, D. E. (1970), *Nature (London) 227*, 837.
Smith, K. D., Church, R. B., and McCarthy, B. J. (1969), *Biochemistry 8*, 4271.
Smith, M. (1963), *Biochem. Biophys. Res. Commun. 10*, 67.
Southern, E. M. (1970), *Nature (London) 227*, 794.
Sueoka, N. (1961), *J. Mol. Biol. 3*, 31.
Taper, H., Wooley, G. W., Teller, M. N., and Lardis, M. P. (1966), *Cancer Res. 26*, 143.
Teng, T. Y., and Sueoka, N. (1964), *Science 143*, 1442.
Waring, M., and Britten, R. J. (1966), *Science 154*, 791.
Yasmineh, W. G., and Yunis, J. J. (1969), *Biochem. Biophys. Res. Commun. 35*, 779.
Yunis, J. J., and Yasmineh, W. G. (1970), *Science 168*, 263.

A Comparison of the Primary Structures of 28S and 18S Ribonucleic Acid from HeLa Cells and Different Human Tissues*

Francesco Amaldi† and Giuseppe Attardi‡

ABSTRACT: The primary structures of 28S and, respectively, 18S RNA from HeLa cells have been compared to those of the high molecular weight rRNA species from different human tissues by several methods at different levels of resolution: base composition analysis, oligonucleotide mapping after pancreatic RNase digestion, analysis of the pattern of degradation by mild digestion with pancreatic or T1 RNase, and RNA–DNA hybridization.

By all these methods no significant difference could be detected between homologous ribosomal RNA components from HeLa cells and various human tissues. It is concluded that the 28S RNA and, likewise, the 18S RNA preparations from different human sources analyzed in the present work are very similar, if not identical. The significance of these results with respect to the problem of the heterogeneity of rRNA genes is discussed.

A redundancy in the genetic information for the two high molecular weight rRNA components and for 5S rRNA has been demonstrated by RNA–DNA hybridization in bacteria and, of two or more orders of magnitude greater, in eukaryotic cells (see review by Attardi and Amaldi, 1970). In HeLa cells, a line of human origin, the number of genes has been found to be about 1000/cell for each of the two high molecular weight components, 28S and 18S RNA (Jeanteur and Attardi, 1969), and about 7600 for the 5S RNA (Hatlen and Attardi, 1971). This redundancy of information raises the problem of the possible variability of the rRNA genes.

The existence of such variability in a higher organism could result either in heterogeneity of the populations of each of the rRNA components within the same cell, or, if all rRNA genes are not equally expressed in different cell types of the same organism, in differences between rRNA preparations from different tissues or developmental stages of the same organism.

Reich *et al.* (1963) had reported substantial differences in base composition between samples of unfractionated rRNA from different tissues of the same animal species, more so than between RNA samples prepared from the same tissues of different animal species. These results, however, were not confirmed by Hirsch (1966), who found the same base composition in 28S RNA, and, respectively, in 18S RNA, purified from various rat and rabbit tissues. Likewise, other investigators could not detect any difference in base composition between preparations of the two high molecular weight rRNA components or of unfractionated rRNA isolated from different developmental stages of the same organism (Lerner *et al.*, 1963; Henney and Storck, 1963; Brown and Gurdon, 1964; Slater and Spiegelman, 1966; Tata, 1967; Grummt and Bielka, 1968). Gould *et al.* (1966) analyzed by polyacrylamide gel electrophoresis the products of limited digestion by T1 ribonuclease of rRNA (unfractionated) prepared from different organisms and from two cell types, reticulocytes and liver cells, of the same organism (rabbit): while they found differences in the pattern of degradation of the rRNA from different organisms, the rRNA from the two rabbit cell types analyzed gave identical results. Similarly, RNA–DNA hybridization experiments failed to show any difference in sequence between high molecular weight rRNA components prepared from rabbit reticulocytes and liver (Di Girolamo *et al.*, 1969), or from different developmental stages of sea urchin (Mutolo and Giudice, 1967).

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† Present address: Centro degli Acidi Nucleici del C. N. R., Istituto di Fisiologia Generale, Università di Roma, Italy.

‡ To whom to address correspondence.