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Sequence Composition of the Template-Active Fraction of Rat Liver Chromatin†

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ABSTRACT: Rat liver chromatin has been separated into nuclease-sensitive and -resistant fractions after mild digestion with DNAase II. The nuclease-sensitive material is further fractionated into Mg^{2+}-soluble and -insoluble chromatin fractions. The kinetics of production of these chromatin fractions have been investigated. After a brief enzyme treatment (5 min at 10 enzyme units/A_{260} unit of chromatin at pH 6.6), 11% of the input chromatin DNA is found in the Mg^{2+}-soluble fraction. This DNA has a weight-average single-strand length of about 400 nucleotides and, as determined by renaturation kinetics, comprises a subset of nonrepetitive DNA sequences and a subset of families of middle repetitive sequences. This demonstrates the nonrandom distribution of repetitive and single copy sequences in the Mg^{2+}-soluble fraction of chromatin. Previous studies have shown that the Mg^{2+}-soluble fraction is enriched in nonrepeated sequences which are transcribed in vivo (Gottesfeld, J. M., Garrard, W. T., Bagi, G., Wilson, R. F., and Bonner, J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71*, 2193–2197). We now report that the Mg^{2+}-soluble fraction of liver chromatin contains a low proportion of sequences in common with the Mg^{2+}-soluble fraction of brain chromatin. Thus, fractionation does not depend on some general property of chromatin but is specific with regard to the template activity of the tissue from which the chromatin was obtained.

Previous work from this laboratory has shown that a minor fraction of DNA in interphase chromatin is more rapidly attacked by the endonuclease DNAase II than is the bulk of chromatin DNA (Marushige and Bonner, 1971; Billing and Bonner, 1972; Gottesfeld et al., 1974a). The amount of DNA in this fraction is variable depending upon the source of the chromatin, but is approximately equal to the proportion of DNA available in a given chromatin preparation for transcription by exogenous RNA polymerase (Billing and Bonner, 1972). The nuclease-sensitive fraction can be separated from the major portion of chromatin by simple procedures based on the solubility of this fraction in either standard saline–sodium citrate (Marushige and Bonner, 1971) or divalent cations (Billing and Bonner, 1972; Bonner et al., 1973; Gottesfeld et al., 1974a). This fraction differs from either unfractionated chromatin or the nuclease-resistant fractions in many respects: namely, chemical composition, chromosomal protein populations, template activity for support of RNA synthesis with exogenous bacterial polymerase, DNA sequence complexity, and DNA sequence homology with cellular RNA (Gottesfeld et al., 1974a). The nuclease-sensitive fraction appears to have the properties expected for transcriptionally active chromatin: it is enriched in nonhistone chromosomal proteins and depleted in histone protein (Marushige and Bonner, 1971; Gottesfeld et al., 1974a, 1975). Nascent RNA is copurified with the Mg^{2+}-soluble fraction of both ascites and HeLa cell chromatin (Billing and Bonner, 1972; Bonner et al., 1975; Pederson and Bhorjee, 1975; Gottesfeld, in preparation) and, most important, the Mg^{2+}-soluble fraction of liver chromatin is enriched in nonrepetitive DNA sequences complementary to cellular RNA (Gottesfeld et al., 1974a). Thus, many lines of evidence support the notion that the nuclease-sensitive, Mg^{2+}-soluble fraction of chromatin corresponds to transcriptionally active regions of chromatin in vivo.

Although active chromatin has been found to differ from inactive regions of chromatin in many respects, the DNA of the Mg^{2+}-soluble fraction is organized in a manner similar to the bulk of the DNA in chromatin; after limited digestion with DNAase II, agarose gel electrophoresis reveals a repeating unit of about 200 base pairs with DNA of the Mg^{2+}-soluble fraction (Gottesfeld and Bonner, 1976). After prolonged nuclease digestion about half of this DNA is rendered acid soluble; furthermore, nuclease-resistant complexes of DNA, RNA, histone, and nonhistone protein have been isolated from Mg^{2+}-soluble chromatin (Gottesfeld et al., 1975). These results are consistent with the finding that active genes are complexed with protein in nuclease-resistant structures (Axel et al., 1975; Lacy and Axel, 1975).

In this report, we expand upon our earlier investigations. From cross-reassociation experiments with the DNAs of the different chromatin fractions, we conclude that fractionation is highly DNA sequence specific. We find that the nuclease-sensitive fraction contains a subset of single-copy sequences and a subset of families of repetitive sequences. Thus, the

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distribution of repetitive and single-copy sequences in the Mg^{2+}-soluble fraction of chromatin is decidedly nonrandom. This finding is significant in light of suggested models of gene regulation (Britten and Davidson, 1971).

Materials and Methods

Preparation and Fractionation of Chromatin. Chromatin from either frozen rat liver (Pel-Freeze, Rogers, Ark.) or rat Novikoff ascites cells was prepared by the method of Marushige and Bonner (1966). Chromatin from frozen rat brain (Pel-Freeze) was prepared by the same method except the crude chromatin was purified by two cycles of sedimentation through 1 M sucrose–10 mM Tris-Cl (pH 8) prior to centrifugation through 1.7 M sucrose. The pellets obtained after ultracentrifugation were washed once in 10 mM Tris-Cl (pH 8) and then dialyzed overnight against at least 200 vol of 25 mM sodium acetate (pH 6.6) at 4 °C. Fractionation was performed as described previously (Gottesfeld et al., 1974a); a brief description of the method is found in the Results section.

Total Rat DNA. DNA was prepared from either crude rat liver or rat ascites chromatin by standard techniques (Marmur, 1961). In the case of ascites DNA, the chromatin was prepared from cells which had been labeled in vivo with [^{3}H]thymidine (Sevall et al., 1975). This DNA was a gift of Dr. J. S. Sevall. DNA was sheared by two passes through the Ribi-Sorvall pressure cell at 50 000 psi. DNA was sheared in moderate salt (either 0.12 M PB[1] or 0.1 M NaCl) to avoid thermal denaturation during this treatment. After shearing, the DNA was extracted once with phenol (saturated with 10 mM Tris-Cl, pH 8) and then with chloroform–isoamyl alcohol (24:1, v/v).

DNA from Chromatin Fractions. DNA from chromatin fraction S2 was prepared in a much gentler manner than described above for total rat DNA. Immediately after fractionation the chromatin was carefully pipetted into a large screw-top test tube containing an equal volume of Tris-saturated phenol. The tube was then laid on its side on the lab bench and incubated at room temperature for 20–30 min. The tube was then gently turned upright such that no mixing of the phenol and aqueous phases occurred. The aqueous phase was removed with a pipet and transferred to another tube above an equal volume of fresh Tris-saturated phenol. This process was repeated 5 more times with phenol and then 2–3 times with chloroform–isoamyl alcohol (24:1, v/v). The final aqueous phase was dialyzed against 10 mM Tris-Cl, 10 mM EDTA, pH 8. Ribonuclease (preincubated at 80 °C for 10 min) was added to 25 μg/ml and the mixture incubated at 37 °C for 1 h. NaCl was added to 0.5 M and sodium dodecyl sulfate to 1%. Pronase (autodigested for 1 h at 37 °C) was added to 25 μg/ml and the mixture was incubated at 60 °C for 1–2 h. Phenol and chloroform extractions were repeated as described above. The final aqueous phase was dialyzed against 10 mM ammonium acetate and then examined by electron microscopy by the aqueous ammonium acetate method of Davis et al. (1971). The bulk of the DNA from any given preparation was not used for electron microscopy and was lyophilized.

DNA from fraction S2 was purified further by chromatography on Sephadex G-200 in either 0.1 N NaOH or 50 mM Na_3PO_4 (Britten et al., 1974). The excluded fractions were pooled, neutralized, dialyzed against ammonium acetate, and then lyophilized. In an early experiment it was found that without G-200 chromatography fraction S2 DNA (5-min enzyme treatment) reassociated to only 70%. It was found that approximately one-third of the DNA applied to a G-200 column (as measured by A_{260}) was included and hence was less than 100–150 nucleotides in length.

DNA from chromatin fractions P1 and P2 was sheared in the Ribi-Sorvall pressure cell as described above for total rat DNA.

Middle Repetitive DNA. The middle repetitive component of either rat liver or ^{3}H-ascites DNA was prepared in the following manner: DNA was dialyzed against ammonium acetate, lyophilized, and redissolved in an appropriate volume of 0.12 M PB. The DNA solution was placed in a Reacti-Vial, denatured in a boiling water bath for 5 min, and incubated to a C_0t of 0.1. Nonreassociated DNA was separated from duplex-containing DNA on hydroxyapatite (Britten and Kohne, 1968). The single-stranded fraction was dialyzed against ammonium acetate, lyophilized, and redissolved in 0.12 M PB. The DNA was incubated to a C_0t of 50, and double-stranded DNA was isolated by hydroxyapatite chromatography. The final duplex-containing material was termed middle repetitive DNA. After this procedure, the single-strand length of the DNA was determined to be 300 nucleotides (determined by alkaline sedimentation velocity, see below). The specific activity of the ascites middle repetitive DNA was 10 000 cpm/μg. Rat liver middle repetitive DNA was labeled in vitro with ^{125}I by the Commerford method (1971) as modified by our laboratory (Holmes and Bonner, 1974). Specific activities of 1–10 × 10^6 cpm/μg were obtained.

Middle repetitive DNA of fractions P1 and S2 were prepared in a manner similar to that described for whole rat DNA except different C_0t values were used. Highly repetitive DNA was removed from sheared P1 DNA after incubation to C_0t 0.05. The single-strand fraction from a hydroxyapatite column was then allowed to reassociate to C_0t 20 and the double-strand fraction was collected. Highly repetitive DNA was removed from S2 DNA after incubation to C_0t 0.02, and middle repetitive DNA was isolated after incubation to C_0t 2. These middle repetitive DNAs were labeled in vitro by nick translation with DNA polymerase I. The following methods were suggested to us by Mr. Glenn Galau and Dr. Francine Eden. Prior to labeling, P1 middle repetitive DNA was incubated to C_0t 55 (without denaturation before incubation); S2 middle repetitive DNA was incubated to C_0t 12. These C_0t values are approximately 250–300 times the $C_0t_{1/2}$ values calculated for pure middle repetitive DNAs. After incubation, the DNAs were dialyzed at 4 °C against 4 l. of 50 mM PB, 1 mM EDTA, pH 7.55 (Schachat and Hogness, 1973). For each sample to be labeled, 0.4 mCi of [^{3}H]TTP (45 Ci/mmol, Nuclear Dynamics) plus 10 μmol of each of dATP, dCTP, and dGTP were lyophilized in a conical centrifuge tube. To this, 2–5 μg of DNA (in 0.25 ml of 50 mM PB, 10 mM $MgCl_2$, 1 mM EDTA, pH 7.55) was added. The reaction was started by the addition of 5 units of DNA polymerase I (Boehringer-Mannheim) per μg of DNA. After incubation at 12 °C for 22–27 h, the reaction was stopped by the addition of EDTA (pH 8) to 50 mM and chilling on ice. Approximately 3 ml of 0.03 M PB–0.1 M NaCl was added; the sample was denatured at 97 °C (3–5 min) and applied directly to a 1-ml hydroxyapatite column equilibrated in 0.03 M PB–0.1 M NaCl. The column was washed with 0.03 M PB–0.1 M NaCl until the effluent contained less than 1000 cpm/20 μl (when counted in 5 ml of Aquasol). The column was then eluted with 0.12 and 0.48 M PB. About 30% of the radioactivity incorporated into DNA eluted in the 0.48 M PB fraction; this material was discarded. Specific activities of 0.5–3.5 × 10^6 cpm/μg DNA were obtained. Labeled middle

[1] Abbreviations used are: C_0t, product of DNA concentration (in mol/l. of nucleotides) and time (in s); PB, phosphate buffer prepared from equimolar amounts of mono- and dibasic sodium phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.

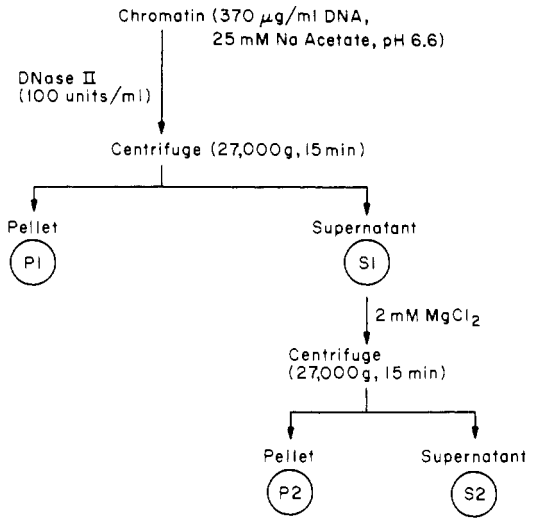

FIGURE 1: Fractionation scheme.

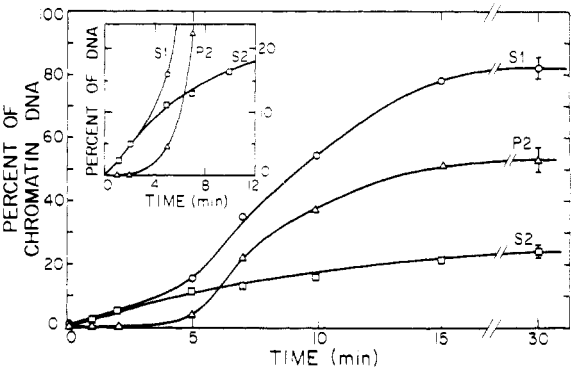

FIGURE 2: Time course of fractionation with DNAase II. Chromatin was fractionated as described (Results) and the amount of nucleic acid in each fraction determined by absorption at 260 nm of an aliquot diluted in 0.9 N NaOH.

repetitive DNA of fraction S2 was further purified by chromatography on Sephadex G-200 in 0.05 M Na_3PO_4. The excluded fractions were pooled, dialyzed against 0.01 M ammonium acetate, and lyophilized.

Further Treatment of Tracer Preparations. In order to remove nonhybridizable material, some tracer preparations (labeled with ^{125}I) were purified on Sephadex G-200. In some cases, foldback or zero-time binding DNA was stripped from the ^{125}I-labeled DNA preparations. This was accomplished by denaturing DNA in 0.03 M PB and pipetting the denatured DNA directly onto a hydroxyapatite column equilibrated in 0.03 M PB–0.1 M NaCl at 60 °C. The time for absorption onto the resin (1–2 min) was such that equivalent C_0t values of no greater than 10^{-4} were obtained. The column was washed with 0.03 M PB–0.1 M NaCl, and the single-stranded DNA was eluted with 0.12 M PB.

DNA Reassociation Kinetics. Kinetics of DNA reassociation were monitored by estimation of duplex formation via hydroxyapatite chromatography (Britten and Kohne, 1968). DNA preparations dissolved in PB were sealed in glass capillary tubes, denatured in a boiling water–ethylene glycol bath (1:1, v/v), and allowed to incubate for various times. Incubations were carried out at 24 °C below the T_m for rat DNA at the particular sodium ion concentration used. The T_m was estimated by the equation of Mandel and Marmur (1968). For 0.72 M Na^+ (0.48 M PB) the T_m of rat DNA is 96 °C and incubations were carried out at 72 °C; for 0.18 M Na^+ (0.12 M PB) the T_m is 86 °C and incubations were at 62 °C; for 0.075 M Na^+ (0.05 M PB) the T_m is 80 °C and incubations were at 56 °C. All C_0t values reported herein have been normalized to C_0t values equivalent to those obtained under standard conditions (0.12 M PB, 62 °C). Normalization of the data was performed according to Britten et al. (1974). After incubation, the contents of the capillary tubes were diluted into 0.03 M PB–0.1 M NaCl, and the solution was applied to a hydroxyapatite column equilibrated in the same buffer at 60 °C. Approximately 1 ml of packed DNA-grade hydroxyapatite (Bio-Rad Bio-Gel HTP) was used for each 100 μg of DNA applied to the column. The column was washed with 4–6 column volumes of 0.03 M PB–0.1 M NaCl; single-strand DNA was eluted with 0.12 M PB and duplex-containing DNA was eluted with 0.48 M PB. An extinction coefficient of 6600 was used for double-stranded DNA and 8850 for single-stranded DNA.

DNA-Excess Reactions. Reassociation experiments, where unlabeled DNA was used in excess over a labeled DNA tracer, were carried out in a manner similar to that described above. Labeled tracer and driver DNAs were incubated in 0.03 M PB plus NaCl to the desired final sodium ion concentration. Hydroxyapatite chromatography was performed as described above. In the case of ^{125}I-labeled DNA tracers, iodine is lost from the DNA during thermal denaturation and incubation at high temperatures. The free iodine in the reaction mixture is not absorbed to hydroxyapatite, and must be washed from the column with 0.03 M PB–0.1 M NaCl. In most DNA-excess experiments, 1-ml hydroxyapatite columns were used, and two 8- to 10-ml fractions were collected (0.12 M PB and 0.48 M PB). The fractions were chilled on ice; 50 μg of bovine serum albumin was added as a carrier, and nucleic acids were precipitated with 7% Cl_3CCOOH for 10–20 min. The precipitates were collected on Schleicher and Schüll type B6 filters and the filters were washed with cold 7% Cl_3CCOOH followed by 60% ethanol. The filters were dried in a vacuum oven at 60 °C and counted in a toluene-based scintillant.

Estimation of DNA Size. DNA size was determined in any one of three ways. Double-stranded DNA was visualized in the electron microscope after spreading from aqueous ammonium acetate by the method of Davis et al. (1971). Phage φX174 DNA was used as a standard. Single- and double-stranded lengths were determined by sedimentation velocity in the analytical ultracentrifuge by the methods of Studier (1965). Weight-average single-strand lengths were also determined from isokinetic sucrose gradients under alkaline conditions. The gradients were formed according to Noll (1967) with the following parameters: C_{TOP} = 15.9% (w/v); C_{RES} = 38.9% (w/v); V_{mix} = 6.1 ml. Gradients were centrifuged in the SW 50.1 rotor at 48 000 rpm for 16 h at 20 °C. A marker of sheared calf thymus DNA was the generous gift of Ms. Maggie Chamberlin. This DNA had a number-average length of 320 nucleotides, as judged by electron microscopy, and an observed sedimentation value of 5.4 S under alkaline conditions.

Results

Fractionation of Chromatin. Figure 1 illustrates the fractionation scheme used in this work. Sucrose-purified chromatin (Marushige and Bonner, 1966) is incubated with DNAase II at pH 6.6 for various lengths of time, and the reaction is stopped by raising the pH to 7.5 with 0.1 M Tris-Cl, pH 11. With purified DNA as the substrate, DNAase II exhibits maximal activity at pH 4.8, and 6–8% of maximal activity at pH 6.6. At pH 7.5, less than 1% of the pH 4.8 activity is observed. Undigested chromatin is removed from solution by

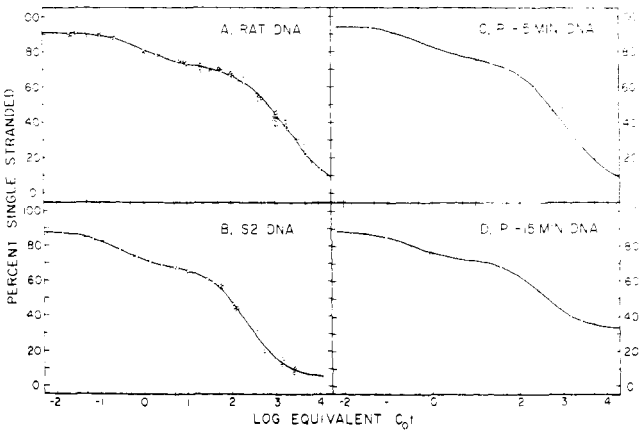

FIGURE 3: Reassociation profile of rat DNA and DNA from the chromatin fractions. Data were obtained at various DNA and PB concentrations and temperatures; however, all data are normalized to C_0t values expected for 0.12 M PB at 62 °C (Britten et al., 1974). The lines through the data were obtained by computer analysis (Britten et al., 1974). (A) Rat DNA. Three sets of data are included: circles are the data of Holmes and Bonner (1974) for rat ascites nuclear DNA; triangles and squares represent the data for two separate preparations of rat liver chromatin DNA. (B) Reassociation profile of fraction S2 DNA isolated after 5 min of nuclease treatment. Chromatin was prepared and fractionated as described. DNA from fraction S2 was subjected to chromatography on Sephadex G-200 in 0.1 N NaOH. The excluded material was dialyzed vs. 0.1 M ammonium acetate, lyophilized, and redissolved in PB. (C) Reassociation kinetics of fraction P1 DNA. Chromatin was incubated with DNAase for 5 min and the DNA from fraction P1 was isolated. This DNA was mechanically sheared to 350 base pairs with the Ribi-Sorvall pressure cell. (D) Reassociation of fraction P1 DNA. Chromatin was incubated with DNAase for 15 min; DNA was isolated from fraction P1 and sheared prior to renaturation.

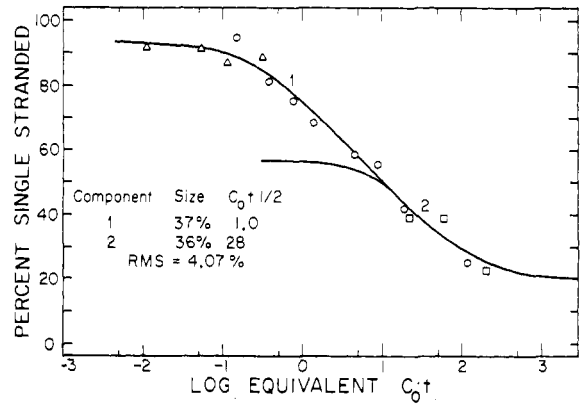

FIGURE 4: Reassociation profile of isolated ^{3}H-labeled middle repetitive rat DNA in the presence of an excess of unlabeled rat DNA. The middle repetitive tracer was isolated from ^{3}H-labeled rat ascites DNA as described (Methods) and mixed with a 200-fold excess of 350 nucleotide-long rat liver DNA in 0.12 M PB (○) or 0.48 M PB (□). The abscissa refers to C_0t values of driver rat DNA. The low C_0t points (△) were obtained in the absence of driver DNA; tracer C_0t values were multiplied by a factor of 5 to correct for the fraction of middle repetitive DNA in whole rat DNA (20%). The line through the data describes a two-component least-squares fit.

centrifugation yielding a pellet (P1). The supernatant (S1) is further fractionated by the addition of $MgCl_2$ to 2 mM. After stirring at 4 °C for 20–30 min, insoluble material which forms is removed by centrifugation. The second pellet is termed P2 and the final supernatant S2.

After 1–2 min of enzyme treatment (inset, Figure 2), all the DNA liberated into fraction S1 is soluble in $MgCl_2$ and is found in fraction S2. On longer times of digestion, Mg^{2+}-insoluble material (P2) is found in fraction S1. On very long times of digestion (ca. 30–90 min), 80% of the chromatin is solubilized and found in fraction S1. The nature of the enzyme resistant 20% has not been fully investigated (Billing and Bonner, 1972). After 30 min of enzyme treatment 20–24% of rat liver chromatin DNA is found in fraction S2. With rat ascites chromatin, 10% of the DNA is found in this fraction (Billing and Bonner, 1972). About 2–5% of duck reticulocyte chromatin DNA is found in fraction S2 (Axel and Felsenfeld, personal communication). Billing and Bonner (1972) have noted a correlation between the amount of DNA in this fraction and the template activity of the input chromatin.

The chemical and physical properties of the chromatin fractions are described elsewhere (Bonner et al., 1973; Gottesfeld et al., 1974a,b 1975; Gottesfeld and Bonner, 1976).

The size of the DNA in fraction S2 was determined after various times of incubation with DNAase II. After 30 s of enzyme treatment, 1.5% of chromatin DNA was found in fraction S2. This DNA had a number-average length of 1800 nucleotide pairs. With longer times of incubation, more DNA is found in fraction S2 and the size of this DNA decreases. The Appendix considers the length of the chromatin segment from which fraction S2 is derived. Long times of incubation with the nuclease (15 min or greater) reduce the size of S2 DNA to below the limits of resolution by electron microscopy. After 15 min incubation, the weight-average length of S2 DNA is approximately 50 base pairs as determined by sedimentation velocity. After 85 min of incubation, 50% of the S2 DNA becomes acid soluble while the remaining 50% is recovered as 120 nucleotide fragments (Gottesfeld et al., 1975).

Kinetic Components of Rat DNA and DNA Isolated from Chromatin Fractions. A reassociation curve of rat ascites nuclear DNA has been presented by Holmes and Bonner (1974). We have obtained similar results with rat DNA prepared from liver chromatin (Figure 3A). In both cases DNA was sheared to a number-average length of 350 nucleotide pairs (as judged by electron microscopy) and reassociation was monitored by retention of duplex-containing DNA on hydroxyapatite. Sheared rat DNA consists of at least three frequency components: a rapidly reassociating component consisting of highly repetitive and foldback sequences (Wilkes and Bonner, in preparation), moderately repetitive sequences which reassociate between C_0t values of 0.05 and approximately 100, and a component of nonrepetitive (single copy) sequences. Second-order reaction curves have been fit to these latter components by the least-squares method of Britten et al. (1974). The parameters describing the moderately repetitive and single copy components of rat DNA are given in Table I.

The intermediate or middle repetitive region of the reassociation curve of rat DNA can be described equally well with either one or two components: the root mean square of a computer analysis with a single middle repetitive component is 3.12% while the root mean square of a two-component analysis is 3.17%. To decide between these two interpretations of the data, we have isolated the middle repetitive sequences of ^{3}H-labeled rat ascites DNA and measured the kinetics of reassociation of this DNA in the presence of an excess of unlabeled rat DNA (Figure 4). The tracer was isolated by standard techniques of reassociation and hydroxyapatite chromatography (Britten and Kohne, 1968). Highly repetitive DNA was removed after incubation to C_0t 0.1; single-stranded DNA was then allowed to reassociate to C_0t 50. The resulting duplex-containing material was used as the middle repetitive tracer. Reassociation takes place over 3–4 log units of C_0t

TABLE I: Kinetic Components of Rat DNA and DNA Isolated from the Chromatin Fractions.

DNA Sample	% of Total Chromatin DNA	Kinetic Component	Fraction of DNA in Component	$C_0t_{1/2}$ Obsd[a]	Estimated $C_0t_{1/2}$ for Pure Component[b]	Average Kinetic Complexity Relative to *E. coli*[c] (Base Pairs)	Repetition Frequency[d]
Unfractionated chromatin DNA	100	Very fast	0.083				
		Intermediate					
		Fast	0.150	0.63	0.095	1.2×10^5	2600
		Slow	0.094	32	3.01	3.8×10^6	50
		Nonrepetitive	0.627	2008	1259	1.6×10^9	1
			rms = 3.17%[g]				
Fraction S2 DNA (5-min nuclease exposure)	11.3 ± 3.9[e]	Very fast	0.109				
		Intermediate					
		Fast	0.165	0.15	0.025	3.2×10^4	1200
		Slow	0.055	2.76	0.15	1.9×10^5	70
		Nonrepetitive	0.628	235	148	1.9×10^8	1
			rms = 1.89%[g]				
Fraction P1 DNA (5-min nuclease exposure)	84.6 ± 4.8[e]	Very fast	0.047				
		Intermediate					
		Fast	0.131	0.54	0.071	9.0×10^4	2600
		Slow	0.175	33	5.8	7.3×10^6	40
		Nonrepetitive	0.608	1706	1037	1.3×10^9	1
			rms = 4.34%[g]				
Fraction P1 DNA (15-min nuclease exposure)	23.9 ± 2.9[f]	Very fast	0.060				
		Intermediate					
		Fast	0.164	0.11	0.018	2.2×10^4	3700
		Slow	0.126	14.8	1.86	2.4×10^6	30
		Nonrepetitive	0.340	593	202	2.6×10^8	1
			rms = 3.95%[g]				

[a] Calculated from a computer analysis of the data of Figures 3A–D (Britten et al., 1974). [b] $C_0t_{1/2}$ for pure component = $C_0t_{1/2}$ observed × fraction of DNA in component. [c] The complexity of *E. coli* is 4.3×10^6 base pairs (Cairns, 1963). The $C_0t_{1/2}$ observed for *E. coli* DNA is 4.1. The difference in base composition of rat (41% GC) and *E. coli* DNA (50% GC) slows the rate of renaturation of rat DNA by a factor of 0.83 (Wetmur and Davidson, 1968) relative to *E. coli*. This assumes that the components of each DNA studied are of the same average base composition as unfractionated rat DNA. [d] Repetition frequency is calculated by dividing the chemical or analytic complexity of each component by the average kinetic complexity observed for that component. Chemical complexity is the total number of nucleotide pairs in a given component, and is obtained from the following expression: $CC = G(fC)(fD)$, where CC is the chemical complexity, G is the genome size of the rat (taken to be 2.1×10^9 base pairs per haploid equivalent of DNA for rodents; Lewin, 1974), fC is the fraction of total chromatin DNA in a given chromatin sample, fD is the fraction of DNA in the kinetic components. [e] Data of Gottesfeld et al. (1974a). [f] Data of Gottesfeld et al. (1974b). [g] Root mean square (rms) deviation of computer analysis (Britten et al., 1974).

(Figure 4), indicating heterogeneity in the population of middle repetitive sequences. A two-component least-squares analysis of the data has been performed (Figure 4), and the $C_0t_{1/2}$ values of the components agree quite closely with the two-component analysis of the middle repetitive region of whole rat DNA reassociation (Table I). We conclude that rat middle repetitive DNA is best described by two components. These components consist of families of sequences which are repeated, on average, 30–70 and 1000–3000 times, respectively, per haploid genome.

Chromatin from rat liver was treated with DNAase II for 5 min and then fractionated as described above (Figure 1). The DNA isolated from fraction S2 was determined to have a number-average double-strand length of 700 nucleotide pairs, as estimated by electron microscopy; however, alkaline sedimentation velocity studies revealed a weight-average single-strand length of 200–600 nucleotides, with a mean of 380 nucleotides (four determinations). S2 DNA was purified by chromatography on Sephadex G-200 prior to use in reassociation experiments.

The data of Figure 3B provide a reassociation curve for fraction S2 DNA of rat liver chromatin. Fractionation was performed after 5 min of enzyme treatment. A second-order fit of the data of Figure 3B has been performed (Britten et al.,

1974), and the parameters describing the kinetic components are presented in Table I. The DNA of this fraction contains four kinetic components in nearly the same proportion as found in DNA of unfractionated chromatin. We do not know whether the rapidly reassociating component of this DNA corresponds to highly reiterated simple sequence DNA or to fragments containing inverted complementary sequences (foldbacks). The intermediately and slowly renaturing components of fraction S2 DNA reanneal at lower C_0t values than their respective counterparts in whole rat DNA. This increased rate of reassociation is not due to differences in fragment length or reaction conditions. Since a random population of DNA sequences would renature with the same kinetics as whole rat DNA, we conclude that fraction S2 represents a specific subset of DNA sequences of the rat genome. This proposition will be substantiated below.

DNA from fraction P1 obtained after 5 min of nuclease treatment was found to have a weight-average single-strand length of 3300 nucleotides (alkaline sedimentation velocity). This DNA was sheared to a number-average length of 350 nucleotide pairs and reassociation kinetics were determined. Fraction P1 DNA and unfractionated chromatin DNA reanneal with similar kinetics (Figure 3C and Table I). This is reasonable since fraction P1 contains 80–85% of the input DNA.

After 15 min of nuclease treatment, fraction P1 contains 24% of whole chromatin DNA. This amount does not decrease after up to 1.5 h of incubation with DNAase. DNA prepared from fraction P1 (15 min nuclease exposure) was found to have a number-average double-strand length of 800 nucleotide pairs. This DNA was sheared to a number-average length of 350 base pairs and reassociation kinetics were determined (Figure 3D and Table I). About 30% of the DNA failed to reassociate, presumably due to degradation caused by the extensive DNAase treatment. The middle repetitive and nonrepetitive components of P1 DNA reassociate 2–5 times faster than their respective counterparts in whole rat DNA. This suggests that fraction P1 obtained after 15 min of nuclease treatment contains a subset of whole genomal DNA sequences; however, further experiments will be needed to confirm this.

Cross-Reassociation Experiments. To test the degree of homology in DNA sequence between the chromatin fractions, we have performed cross-reassociation experiments. Figure 5 depicts the reassociation of labeled P1 DNA in the presence of an excess of either unfractionated chromatin DNA or fraction S2 DNA. These DNAs were obtained from chromatin fractions after 5 min of nuclease treatment. When driven by whole chromatin DNA, the labeled P1 DNA tracer reassociates with kinetics essentially identical with the kinetics of the driver. On the other hand, when fraction S2 DNA is used as the driver (upper curve, Figure 5) reassociation is limited to 28% at a driver C_0t of 3000. By this C_0t, 90% of S2 DNA has reassociated (Figure 3B). As much as one-half of the observed P1 tracer reassociation could be due to self-reaction since an S2 driver C_0t of 3000 corresponds to a tracer C_0t of 0.48 (C_0t 3000 divided by the driver to tracer mass ratio). A $C_0t_{1/2}$ of 600 is observed for a single transition in the P1 DNA tracer reassociation. This should be compared to a driver $C_0t_{1/2}$ of 225 for the nonrepetitive portion of S2 DNA reassociation. These data suggest that most (60–70%) of the DNA sequences present in fraction P1 are absent from fraction S2. It is possible, however, that the reassociation of P1 tracer has not terminated by C_0t 3000 of the S2 driver DNA. If further reassociation was observed at higher C_0t values, this would indicate that P1 sequences are found in low abundance in S2 DNA. With this

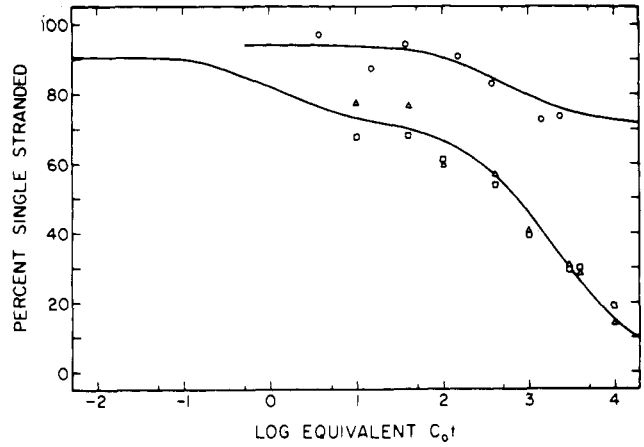

FIGURE 5: Cross-reassociation of S2 and P1 DNAs. Chromatin was incubated with DNAase II for 5 min and fractionated, and DNA prepared from the fractions as described. Fraction P1 DNA was labeled in vitro with ^{125}I (Commerford, 1971; Holmes and Bonner, 1974). Fast reassociating material was stripped from the labeled DNA (Methods). In the lower curve ^{125}I-P1 DNA (□) was mixed with total rat liver chromatin DNA (△) at a driver to tracer ratio of 12 800:1. In the upper curve ^{125}I-P1 DNA (○) was mixed with fraction S2 DNA (5-min nuclease treatment) at a driver to tracer ratio of 6250:1. The abscissa refers to C_0t values normalized to standard conditions (0.12 M PB, 62 °C) for the concentration of driver DNAs. The line through the data of the lower curve is the computer fit of the reassociation curve for rat DNA (Figure 3A). The upper curve represents a least-squares fit of one second-order component. The root mean square of the fit is 3.4% and the $C_0t_{1/2}$ value observed is 600; the reaction terminates at 28% reassociation with 23% reassociation in this single component.

reservation in mind, we may still conclude that fractionation according to DNA sequence has been accomplished by the methods employed (Figure 1).

When fraction S2 DNA was used as a tracer and fraction P1 DNA was used as a driver, very different results were obtained. The S2 DNA tracer reassociated to the extent of 70% when unfractionated chromatin DNA ($C_0t = 10^4$) or fraction S2 DNA ($C_0t = 5 \times 10^3$) was used as a driver. Fraction P1 DNA was isolated after 5 mln of nuclease treatment. When this DNA was the driver, 53% reassociation of the S2 tracer was observed at a similar driver C_0t (10^4). The $C_0t_{1/2}$ values for the slow components of both tracer and driver DNAs were comparable (1.07×10^3 and 0.86×10^3). These data indicate that, after 5 min of nuclease treatment, most S2 DNA sequences (≥75%) are still found in fraction P1. In agreement with this finding, Figure 2 shows that not all S2 chromatin is separated from P1 chromatin by fractionation after a 5-min exposure to DNAase.

To determine the degree of tissue specificity in the DNA sequences of fraction S2, we isolated fraction S2 from both liver and brain chromatin. The chromatin was treated with DNAase II for 5 min and fractionated, and DNA was prepared. S2 DNA of brain chromatin was labeled in vitro with ^{125}I (Commerford, 1971; Holmes and Bonner, 1974). The labeled brain S2 DNA reassociated to 82% with whole rat DNA as a driver (at a driver C_0t of 10^4). On the other hand, when liver S2 DNA was the driver, the reassociation kinetics of Figure 6 were obtained. Brain S2 DNA reassociated to only 31% at a driver C_0t of 2000. At this C_0t, the liver S2 driver DNA reassociated to 90% (lower curve, Figure 6). A computer analysis of the data suggests that brain S2 DNA might reassociate to 40% at higher C_0t values ($>10^4$); however, we have no data to support this. Furthermore, control experiments show that 10% out of the observed 30% reassociation at C_0t 2000 could be due to tracer self-reaction. We take these data to mean

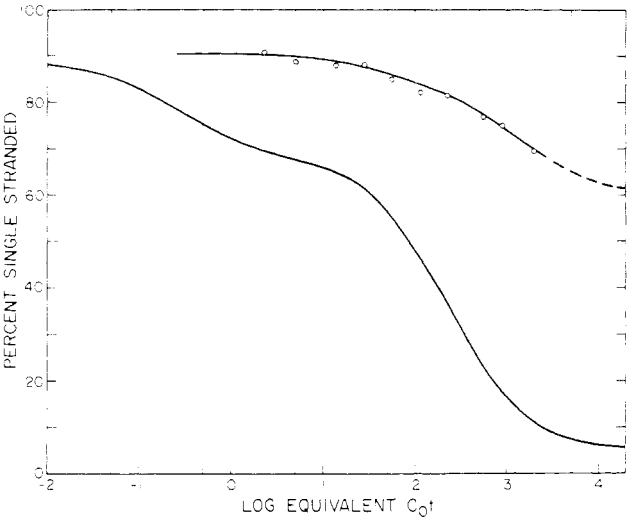

FIGURE 6: Cross-reassociation of brain and liver fraction S2 DNAs. Chromatin was prepared from brain and liver and fractionated, and DNA isolated as described. Incubation with DNAase II was for 5 min. Brain S2 DNA was labeled with ^{125}I, and the labeled DNA was mixed with unlabeled liver S2 DNA at a liver DNA to brain DNA ratio of about 2000:1. Both DNAs were chromatographed on Sephadex G-200 (0.1 N NaOH) and the excluded material was utilized in these experiments. Incubations were in 0.48 M PB (72 °C). The abscissa refers to C_0t values of driver liver S2 DNA, corrected to 0.12 M PB (62 °C). The lower curve is a reassociation profile of liver S2 DNA (Figure 3B); the upper curve is a least-squares fit of the data (root mean square = 0.8%). The computer fit describes two components, one (7.4%) with a $C_0t_{1/2}$ of 50 and another (23.9%) with a $C_0t_{1/2}$ of 1500. The extrapolated final reaction is 41%.

that chromatin fractionation is decidedly tissue specific; we estimate that no more than 25–40% of the sequences of brain S2 DNA are present in the major frequency components of liver S2 DNA.

Further Studies on the Kinetic Components of Fraction S2 DNA. We indicated earlier that the slow component of fraction S2 DNA (Figure 3B) corresponds to nonrepetitive sequences. This can be shown directly by isolating this component by hydroxyapatite chromatography, labeling the DNA in vitro, and reannealing in the presence of an excess of unlabeled whole rat DNA (Figure 3 of Gottesfeld et al., 1974a). The labeled DNA reassociated with only the nonrepetitive sequences of the driver DNA. From the reassociation profile of fraction S2 DNA (Figure 3B), it can be calculated that if this fraction represents a specific subset of the rat genome (11.3%), then the slow component reassociates at the rate predicted for nonrepeated sequences (Table I). We may therefore conclude that since the slow component is derived from the nonrepeated sequences of whole rat DNA, and since it reassociates 8.5 times more rapidly than whole rat DNA, fraction S2 must represent 11–12% of the complexity of whole rat nonrepetitive DNA.

We next turn attention to the moderately repetitious DNA of fraction S2. The computer analysis of the reassociation curve of S2 DNA (Figure 3B, Table I) suggests that the intermediate frequency components comprise a limited fraction (5–27%) of the complexity of whole rat middle repetitive DNA. To obtain a more precise estimate of this kinetic enrichment, we have isolated the middle repetitive DNA of fractions S2 and P1 and determined the kinetics of reassociation of these DNAs (Figure 7). The middle repetitive DNAs were isolated by standard techniques of reassociation and hydroxyapatite chromatography; C_0t values were chosen to minimize contamination with highly repetitive and nonrepetitive sequences (see Methods). The DNAs were labeled in vitro by treatment

TABLE II: Complexity of Middle Repetitive DNA.

DNA of Chromatin Fraction	Complexity of Middle Repetitive Components[a] (Base Pairs)	
	Fast Component (1)	Slow Component (2)
S2	300	110 000
P1	4200	2 400 000

[a] Kinetic complexity calculated relative to *E. coli* (see Table I, footnotes).

with *E. coli* DNA polymerase I, [^{3}H]TTP, and unlabeled deoxynucleoside triphosphates. The labeled DNAs were then mixed with an excess of unlabeled S2 or P1 middle repetitive DNA, and reassociation kinetics were determined (Figure 7).

The reassociation of S2 and P1 middle repetitive DNA takes place over 4–5 log units of C_0t (Figures 7A,C). These data confirm the result of Figure 4; middle repetitive DNA of the rat consists of a spectrum of frequency components. Computer analysis suggests that the reassociation curves are best approximated by two second-order kinetic components. The size of these components and $C_0t_{1/2}$ values are given in Figure 7. The data demonstrate that chromatin fraction S2 contains a smaller proportion of the complexity of whole rat middle repetitive DNA than chromatin fraction P1. From the data of Figures 7A and 7C, we calculate that the middle repetitive complexity of fraction S2 is 5–8% that of fraction P1. The kinetic complexities of the components of P1 and S2 middle repetitive DNAs are listed in Table II. The complexity of the S2 fast-reassociating component borders on the complexity of simple-sequence DNA. This complexity is 7% that of the fast component of P1 middle repetitive DNA. The complexity of the S2 slow repetitive component is 5% that of the P1 slow repetitive component. The slow repetitive component of fraction S2 could accommodate 350 families of sequences each 300 nucleotides in length, while the slow repetitive component of fraction P1 could accommodate nearly 7000 different sequences of this length. The data of Figure 3 and Table I suggest that very slow repetitive components exist in rat DNA. These components are absent from the P1 and S2 repetitive DNA tracers (Figure 7) due to the fractionation steps used in preparing these DNAs (see Methods).

The reassociation experiments of Figures 7B and 7D were performed to estimate the degree of cross-contamination of middle repetitive sequences between the chromatin fractions. When labeled S2 repetitive DNA was allowed to reassociate in the presence of an excess of unlabeled P1 middle repetitive DNA, the data of Figure 7B were obtained. This reassociation curve is similar to the reassociation curve of P1 middle repetitive DNA (Figure 7C). Since the S2 tracer reassociates to the same extent with either S2 or P1 middle repetitive DNA as the driver, we conclude that all sequences of S2 middle repetitive DNA are present in fraction P1. However, the S2 sequences comprise a low percentage of the total complexity of P1 middle repetitive DNA (Table II). The reassociation kinetics of Figures 7A and 7B allow us to calculate that S2 repetitive sequences make up between 5 and 14% of the complexity of P1 middle repetitive DNA. This calculation was made by comparing the $C_0t_{1/2}$ values of the kinetic components. If we compare the overall C_0t values of the curves of Figures 7A and 7B, we calculate that S2 sequences comprise 11% of P1 middle repetitive complexity.

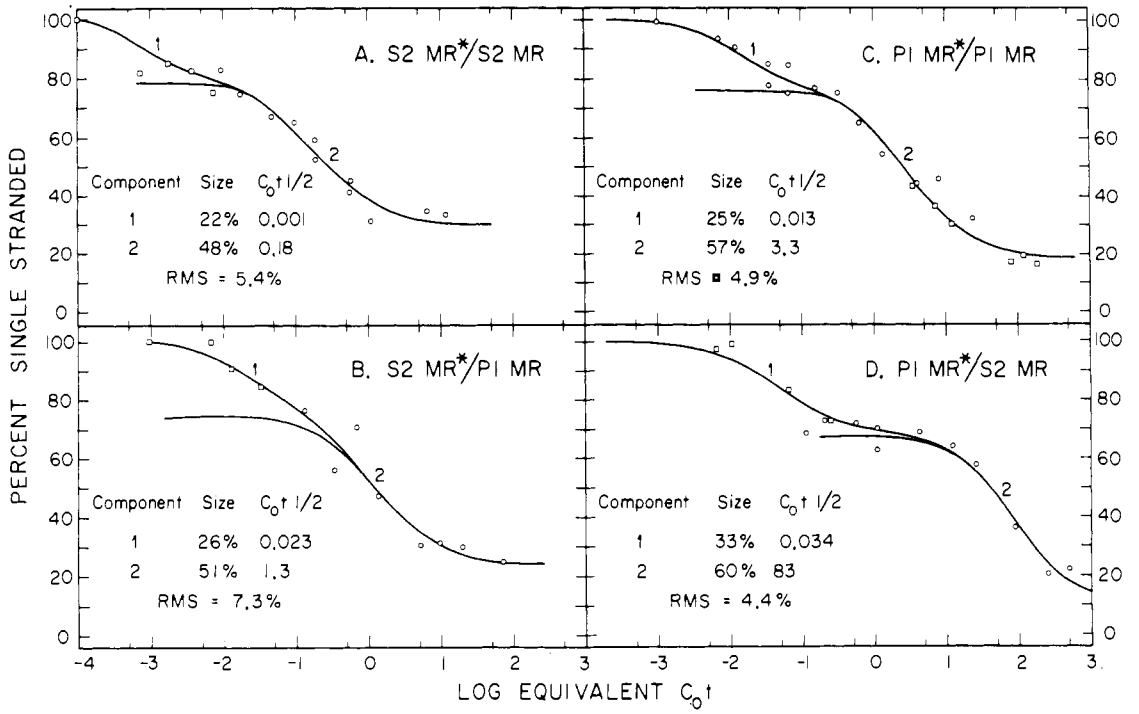

FIGURE 7: Reassociation curves of ^{3}H-labeled middle repetitive DNAs from fractions P1 and S2. (A) Middle repetitive DNA of fraction S2 was labeled by nick translation (Methods) and mixed with unlabeled S2 middle repetitive DNA at driver to tracer ratios of 1200:1 (○) and 5:1 (□). (B) S2 tracer in the presence of unlabeled P1 middle repetitive DNA. The driver to tracer ratios were 2600:1 (○) and 450:1 (□). (C) P1 middle repetitive DNA, labeled by nick translation, was mixed with unlabeled P1 middle repetitive DNA at driver to tracer ratios of 5700:1 (□) and 1000:1 (○). (D) P1 tracer in the presence of S2 middle repetitive DNA. The driver to tracer ratios were 4500:1 (○) and 900:1 (□). All reactions were carried out in 0.12 M PB in sealed capillary tubes. C_0t values refer to the concentration of driver DNAs. The labeled DNAs exhibit 7-8% zero time binding to hydroxyapatite in 0.12 M PB. The data have been corrected for zero time binding according to the equation of Davidson et al. (1973). The lines through the data are the best two-component least-squares analyses (Britten et al., 1974). The component sizes and $C_0t_{1/2}$ values are given for each curve.

The data of Figure 7D allow us to estimate the contamination of P1 repetitive sequences in S2 middle repetitive DNA. In this experiment, labeled P1 DNA was used as tracer and unlabeled S2 DNA was used as driver. Since the tracer reassociates to about the same extent with either P1 or S2 DNA as driver (Figures 7C,D), fraction S2 must contain all P1 sequences. We note, however, that during the last phase of reassociation (at C_0t values >50) tracer self-reaction could contribute significantly to the observed reassociation. Reassociation of the tracer has been divided into two components: one component (33%, $C_0t_{1/2}$ = 0.034) reassociates over the same range of C_0t values as the major portion of S2 driver reassociation (C_0t 0.005-1.0). The second component (60%, $C_0t_{1/2}$ = 83) reassociates nearly 500 times slower than the slow component of the S2 driver ($C_0t_{1/2}$ = 0.18). This suggests that about 60% of P1 sequences are present in the S2 middle repetitive DNA preparation in extremely low concentration (0.18/83 = 0.2%). This could be an underestimate since P1 sequences in S2 DNA might have been partially removed by the C_0t 2 fractionation step used in the preparation of S2 middle repetitive DNA (see Methods). The data of Figure 7D suggest that about one-third of the mass of P1 repetitive DNA has complements in S2 DNA at significant concentrations. The experiment depicted in Figure 7B showed that about 11% of the complexity of fraction P1 repetitive DNA is due to the presence of S2 repetitive sequences. Table III summarizes our knowledge of the distribution of repetitive sequences in fractions P1 and S2. We have indicated our estimates of the degree to which each kinetic component may contribute to the middle repetitive DNA of fractions P1 and S2.

To obtain an estimate of the fraction of total middle repetitive DNA complementary to S2 DNA we performed the experiment of Figure 8. In different experiments ^{3}H-labeled and ^{125}I-labeled middle repetitive DNA from unfractionated chromatin was mixed with an excess of S2 DNA (unfractionated with respect to kinetic components). Figure 4 provides a control for this experiment, the reassociation of ^{3}H-labeled middle repetitive DNA in the presence of an excess of DNA from unfractionated chromatin. The experiment of Figure 4 is described in detail above. When the middle repetitive tracer

TABLE III: Composition of Middle Repetitive DNA of Chromatin Fractions P1 and S2.[a]

	$C_0t_{1/2}$ of Repetitive Component					
	Fast Repetitive			Slow Repetitive		
Chromatin Fraction[b]	0.001	0.013	0.034	0.18	3.3	83
S2	22%	26%	possible 10-20%	48%	51%	0.2-5%[d]
	(7a)[c]	(7b)	(7a)	(7a)	(7b)	(7a)
P1	<2-4%	25%	33%	possible 5-10%	57%	60%
	(7c,d)	(7c)	(7d)	(7c,d)	(7c)	(7d)

[a] % of repetitive DNA in component (by mass); from data of Figures 7A-D. [b] Chromatin fractions were obtained after 5 min of nuclease treatment. [c] Indicates that this estimate was obtained from Figure 7A, etc. [d] 0.2% is an underestimate since some of this component, if present in S2 DNA, would have been lost from isolated S2 middle repetitive DNA by C_0t 2 fractionation (see text).

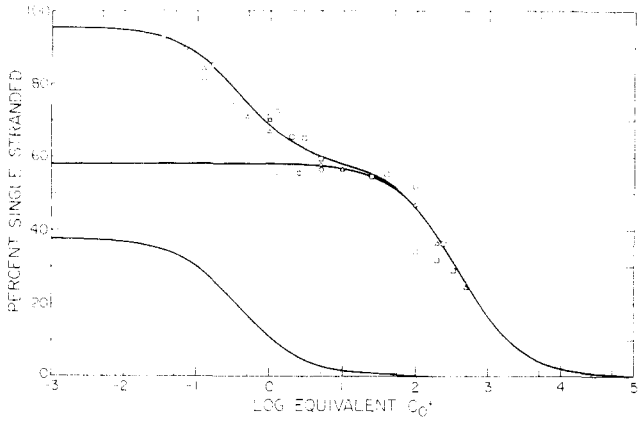

FIGURE 8: Reassociation profile of labeled middle repetitive rat DNA in the presence of an excess of unlabeled fraction S2 DNA. Middle repetitive DNA was prepared from either ^{3}H-labeled ascites nuclear DNA (□) or from unlabeled rat liver DNA and labeled in vitro with ^{125}I (○,△). The ratios of unlabeled S2 DNA to tracer repetitive DNAs were 2000:1 (○), 1400:1 (△), 3000:1 (□). The abscissa refers to S2 DNA driver C_0t values. The line through the data is a two-component least-squares fit (root mean square = 4.5%). Component 1 (37.5%) has an observed $C_0t_{1/2}$ of 0.33, and component 2 (58%) has an observed $C_0t_{1/2}$ of 380.

was used with unlabeled S2 DNA as a driver, the results of Figure 8 were obtained. Data for three separate experiments (three different tracer preparations) are included, and data are normalized to 100% reassociation of the tracer when driven by whole DNA. The reassociation of the repetitive tracer takes place over at least 4 logs of driver C_0t. A two-component least-squares fit of the data is shown in Figure 8.

On first inspection these data indicate that all sequences of middle repetitive DNA are present in fraction S2; however, since the reaction proceeds over several decades of C_0t, the families of middle repetitive sequences are present at various levels of abundance in S2 DNA. From the fit of the data (Figure 8), 37% of the hybridizable tracer reassociates over the same range of C_0t values as the intermediate components of fraction S2 DNA (C_0t 0.02–10). The second component (58%) exhibited a $C_0t_{1/2}$ of 380. This value is nearly the $C_0t_{1/2}$ value for nonrepetitive DNA in the S2 driver DNA ($C_0t_{1/2}$ = 225, Table II). We interpret these data to mean that 37% of the mass of rat middle repetitive DNA has complements in S2 DNA in high abundance (i.e., 100–2000 copies/haploid genome), while the major fraction of repetitive sequences (58%) is represented only once or a few times in fraction S2 DNA.

Discussion

Over the past decade several attempts have been made to fractionate interphase chromatin into transcriptionally active and inactive segments. The procedures described to date start with sonicated or pressure-sheared chromatin, and fractionation is performed either by differential sedimentation (Frenster et al., 1963; Murphy et al., 1973) or by chromatography (McConaughy and McCarthy, 1972; McCarthy et al., 1973; Simpson and Reeck, 1973). We have chosen to take another approach (Marushige and Bonner, 1971): chromatin DNA is digested with the endonuclease DNAase II under conditions of low enzymatic activity for brief periods of time. Fractionation is achieved in two ways: first, DNAase II appears to attack the transcriptionally active regions of chromatin more readily than transcriptionally inactive chromatin (Figure 2); second, inactive chromatin can be removed from solution by selective precipitation with either saline–citrate (Marushige and Bonner, 1971) or divalent cations (Billing and Bonner, 1972). Using added radioactive polynucleotides, we find no detectable protein rearrangement during either enzyme treatment or subsequent fractionation (Gottesfeld et al., unpublished). We reported previously (Gottesfeld et al., 1974a) that the active fraction S2 is enriched in nonhistone chromosomal proteins and depleted in histone protein. Furthermore, nascent RNA appears to cofractionate with S2 chromatin (Bonner et al., 1975). We have also found that the circular dichroism spectrum of the active chromatin fraction is more like the spectrum of "B"-form DNA than the spectra of either unfractionated chromatin or the inactive fractions (Gottesfeld et al., 1974b). Similarly, Polacow and Simpson (1973) have reported that the active fraction obtained by ECTHAM chromatography of sonicated chromatin is in an extended DNA-like conformation. Recent evidence suggests that the DNA of both active and inactive regions of chromatin is organized into repeating nucleoprotein subunits (Axel et al., 1975; Lacy and Axel, 1975; Gottesfeld et al., 1975). The subunits of inactive chromatin appear to be complexes of DNA and histone (Kornberg, 1974), while the subunits of Mg^{2+}-soluble active chromatin are composed of DNA, RNA, histone, and nonhistone protein (Gottesfeld et al., 1975).

We have shown previously (Gottesfeld et al., 1974a) that the nuclease-sensitive, Mg^{2+}-soluble fraction of liver chromatin is enriched four- to fivefold in nonrepetitive DNA sequences which are transcribed in vivo. This was demonstrated by RNA-excess hybridization with labeled nonrepetitive DNA as a tracer. We estimated that as much as 29% of the hybridizable tracer DNA from fraction S2 forms DNA–RNA hybrids (Gottesfeld et al., 1974a). This suggests that fraction S2 is 58% "pure" in transcribed nonrepetitive sequences. If the extent of nonrepetitive transcription in the liver is 6–10% of the double-strand complexity (Grouse et al., 1972; Brown and Church, 1972), then we have purified transcribed sequences by a factor of 6–10 ((58/10)–(58/6)) over unfractionated chromatin. It is likely that fraction S2 codes primarily for nuclear RNA rather than messenger RNA. Nuclear RNA represents about 10% of the total double-strand complexity of the rat genome in ascites cells (Holmes and Bonner, 1974). In contrast, messenger RNA complexities tend to be about one-tenth that of nuclear RNA (Galau et al., 1974; Davidson and Britten, 1973; Hough et al., 1975; Holmes and Bonner, unpublished). We are currently investigating the distribution of single-copy sequences complementary to polysomal mRNA in the chromatin fraction.

Cross-reassociation experiments with fraction S2 DNA from both liver and brain chromatin demonstrate that a low proportion of brain S2 sequences are present in the liver S2 fraction (Figure 6). We believe, therefore, that our fractionation scheme results in the isolation of a tissue-specific transcribed portion of chromatin. We conclude that fractionation does not depend on some general property of chromatin, but rather on the transcriptional state of the genome in the particular cell type under study.

From the kinetics of reassociation of fraction S2 DNA (Figure 3B), we conclude that this fraction represents a specific subset of sequences of the rat genome. Knowing that the slow kinetic component of S2 DNA is derived from nonrepeated sequences (Gottesfeld et al., 1974a), we calculate that this component comprises 11% of the total single-copy complexity of the rat. The data of Figure 3B also suggest that fraction S2 contains a subset of repeated sequences. This notion is substantiated by the data of Figures 7A and 7C: the complexity of S2 middle repetitive DNA is about 5–8% that of P1 middle repetitive DNA (Table II). Similarly, the cross-reassociation experiment of Figure 7B suggests that S2 sequences comprise

approximately 11% of the complexity of P1 middle repetitive DNA; the kinetics indicate that S2 sequences are in nearly the same or slightly greater abundance as the majority of P1 middle repetitive sequences. Two alternative explanations for the presence of S2 sequences in substantial concentration in fraction P1 are possible: first, the fractionation procedure used (Figure 1) does not remove all S2 chromatin from fraction P1 after 5 min of nuclease treatment (Figure 2). Second, S2 repetitive sequences may be localized in both active and inactive regions of chromatin, and fractionation would never remove these sequences from P1 chromatin. Nevertheless, it is clear that fraction S2 contains at most one-tenth of the repetitive sequence complexity of fraction P1 (Table II).

The experiment of Figure 7D allows us to estimate the purity of S2 repetitive DNA. One-third of the mass of P1 repetitive DNA can form duplexes with S2 repetitive DNA over the C_0t values at which S2 DNA reassociates (C_0t 0.005–1). The remaining P1 repetitive DNA reassociates at much higher C_0t values, indicating that these sequences are present in S2 DNA in extremely low concentration. We calculated earlier that S2 sequences make up 11% of the complexity of P1 repetitive DNA; the kinetics of Figure 7 suggest that S2 sequences might contribute more to the mass of P1 DNA than to the complexity. We calculate that S2 sequences could contribute 20% of the mass of P1 repetitive DNA. Thus, an additional 10–20% of the P1 repetitive complexity is found in S2 DNA in significant abundance. Most P1 repetitive sequences are present in S2 repetitive DNA in very low concentration—0.2% of the concentration of S2 slow component sequences. This is probably an underestimate; the C_0t 2 fractionation step used in preparing S2 repetitive DNA could have reduced the concentration of P1 sequences in S2 DNA. We calculate that these P1 repetitive sequences could be present in S2 DNA at 5% of the abundance of S2 repetitive sequences. In summary, we have shown that fraction S2 consists of a highly selected population of DNA sequences. The major fraction of the mass of S2 DNA (ca. 70–90%) comprises no more than 10–12% of the complexity of the rat genome.

Our finding of limited repetitive and single-copy complexities in fraction S2 DNA suggests that the distribution of repetitive and nonrepetitive sequences in the genome is nonrandom. Furthermore, fraction S2 is enriched in transcribed single-copy sequences. This suggests that specific repetitive sequences might lie adjacent to transcribed nonrepetitive sequences (Bishop and Freeman, 1974; Davidson et al., 1975b). At present, we do not know to what extent the repetitive sequences of fraction S2 are transcribed. Britten and Davidson (1971) have postulated that the control of transcription in eukaryotes is mediated through the binding of activator molecules to sites on repetitive DNA, promoting the transcription of neighboring sequences. This model and its consequences have been reviewed recently by Davidson and Britten (1973). The model would predict that, associated with transcribed single-copy sequences, one should find a limited complexity of repetitive sequences. The results reported herein are consistent with the predictions of the Britten–Davidson model.

In the Appendix we show that the chromatin DNA segment from which fraction S2 is derived is, on average, 6000–7000 nucleotide pairs in length. This is three–five times the length expected for a DNA sequence which codes for a messenger RNA of average length (Davidson and Britten, 1973), and it approaches the length of nuclear RNA molecules (Holmes and Bonner, 1973; Davidson and Britten, 1973). At present we do not know whether the entire length of an S2 chromatin segment is transcribed. The hybridization experiments cited above (Gottesfeld et al., 1974a) suggest that 29% of fraction S2 DNA hybridizes to a 20 000-fold excess of whole cellular RNA. Assuming that transcription is asymmetric, this corresponds to 58% of fraction S2 DNA being transcribed. Why is this figure not 100%? From the complexity measurements on fraction S2 DNA it is highly unlikely that 42% of the mass of S2 DNA could be due to contamination with random DNA sequences. Perhaps there are specific nuclease-sensitive nontranscribed sequences adjacent to the transcribed sequences of fraction S2. Perhaps some transcribed RNAs are digested by RNAase so rapidly that they are not present in the cellular RNA preparation in sufficient amounts to drive the hybridization reaction. These are matters for further investigation.

Acknowledgment

We wish to thank Mr. Mahlon Wilkes for performing the electron microscopy for us, and Mr. Ralph Wilson for technical assistance during the early portion of this work. We are very grateful to Drs. Roy Britten, David Holmes, Barbara Hough, and William Klein, and to Mr. Glenn Galau for numerous discussions and evaluation of this work.

Appendix: *Theory for the Amount and Fragment Length of Template Active Chromatin Liberated by Double-Strand Cuts*

Norman Davidson, William Pearson, Joel Gottesfeld, and James Bonner*

The experiments reported in the main paper suggest that chromatin consists of template-inactive regions that are relatively resistant to DNAase II and interspersed template-active segments that are sensitive to double-strand endonucleolytic cuts by DNAase II. This model implies that two double-strand endonucleolytic cuts are needed for a fragment of the template-active segment to be released into the soluble (S2) fraction. We present an analysis suggesting that the average length of a template-active segment is 6500 ± 500 nucleotide pairs and that the fraction of the genome which is template active is 0.20 ± 0.03.

The data that we wish to analyze deal with the average length of and the amount of DNA released into fraction S2 as a function of time of digestion (see Figure A1 of this appendix and Figure 2 of the main paper). Note that the average length of the released DNA is 1800 nucleotide pairs after 30 s of digestion and the length decreases with increasing time of digestion. The amount released into S2 increases with time of digestion and levels off at about 20%.

Let the average length of a template active segment be L_0. Assume that DNAase II acts by introducing double-strand breaks into the template-active segments. Template-active DNA that is attached to inactive DNA remains in the insoluble (P1 and P2) fractions. A fragment of template-active DNA that is detached from the inactive material by endonucleolytic cuts on each side is in the soluble (S2) fraction. We assume that these cuts are distributed at random within the template-active segments. Clearly, the average length of the released template-active segments will decrease as a function of the number of endonucleolytic events, and the total amount of material detached from the template inactive regions will increase with the amount of digestion.

Suppose j double-strand cuts are made at random in a segment of length, L_0, bounded by the insoluble template-inactive regions. It is intuitively obvious (and can be confirmed by a

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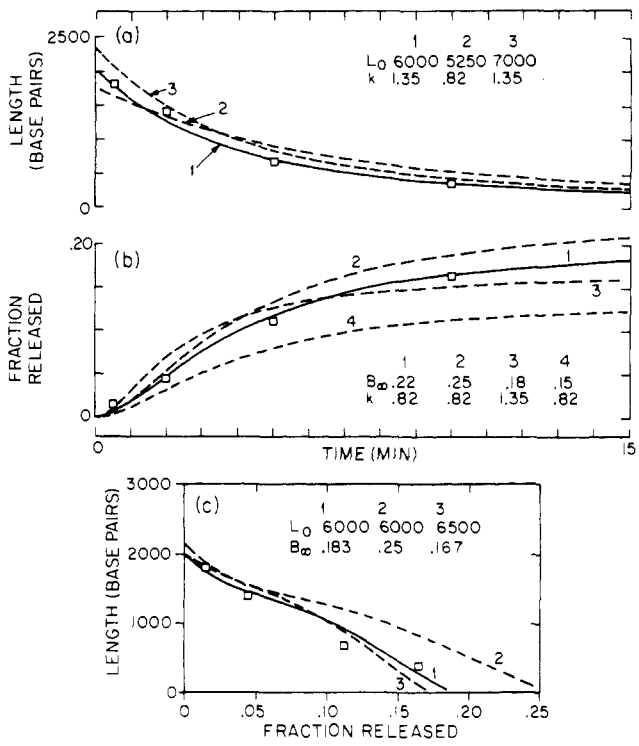

FIGURE A1. Length of DNA fragments and fraction released from chromatin with time of DNAase II digestion. The curves describe the best computer fit (curve 1, solid line) and alternative values of the parameters L_0 (active segment length in base pairs), B_∞ (fraction of DNA), and k (nucleolytic cuts segment^{-1} min^{-1}) in the equations describing the digestion (eq A4, A5). (a) Number-average length of DNA in fraction S2 as a function of nuclease digestion time. Double-strand DNA lengths were determined by electron microscopy. Curve 1 is the best least-squares fit of the parameters L_0 and k in eq A4 to the experimental data (□). Curve 2 is a plot using the value of the parameter k determined from the best fit in Figure A1b. Curve 3 is a plot indicating an upper limit for the parameter L_0. (b) Fraction of chromatin DNA released into S2 with time of DNAase II digestion. Curve 1 is the best least-squares fit of the parameters B_∞ and k in eq A5 to the data (□, Figure 2 of main paper). Curve 3 is a plot of the equation using the parameter k determined from the best fit in Figure A1a. Curves 2 and 4 show the variation of the fit with parameter B_∞. (c) Length of DNA released as a function of the fraction of DNA released (a replot of the data from (a) and (b) to remove time). Curve 1 shows the best least-squares fit of the parameters L_0 and B_∞ to a curve generated by plotting eq A4 vs. A5. Curve 2 shows the fit with a large value of B_∞, while curve 3 shows the range of values for L_0 and B_∞ similar to the best fit.

formal algebraic calculation) that these cuts divide the template-active segment into $j + 1$ fragments, each of average length $L_0/(j + 1)$. Thus, the fragments released by the first two cuts have an average length of $L_0/3$.

Let the average number of cuts per template-active segment be n. The probability that any one active segment will have j cuts is given by the Poisson distribution:

$$P_n(j) = \frac{n^j e^{-n}}{j!} \tag{A1}$$

Let B_∞ be the fraction of the genome which is template active and B_n the amount released after an average of n nucleolytic cuts/segment. For a particular segment of length L_0 which has received j cuts, $(j - 1)$ fragments, each of average length $L_0/(j + 1)$, are released. Therefore, the fraction of this segment released is $(j - 1)/(j + 1)$. As stated above, the probability of j cuts is $P_n(j)$. Therefore, the fraction of the entire genome released is:

$$(B_n/B_\infty) = \sum_{j=2}^{\infty} [(j - 1)/(j + 1)]P_n(j) \tag{A2}$$

There are $(j - 1)$ fragments, each of average length $L_0/(j + 1)$ released from a segment which received j cuts. The total number of fragments released per template-active segment, if there is an average of n cuts/segment, is $P_n(2) + 2P_n(3) + \ldots (j - 1)P_n(j)$ or $\Sigma^{\infty}_{j=2} (j - 1)P_n(j)$. Therefore, $\bar{L}_n$, the number-average length of the fragments released after n cuts, is given by

$$\bar{L}_n = \frac{\sum_{j=2}^{\infty} [(j - 1)L_0/(j + 1)]P_n(j)}{\sum_{j=2}^{\infty} (j - 1)P_n(j)} \tag{A3}$$

By algebraic manipulations one then derives

$$(\bar{L}_n/L_0) = \frac{e^n - (2e^n/n) + (2/n) + 1}{e^n(n - 1) + 1} \tag{A4}$$

and

$$B_n/B_\infty = e^{-n}[e^n - (2e^n/n) + (2/n) + 1] \tag{A5}$$

These functions have the following limiting behavior as $n \to 0$.

$$(L_n/L_0) \to \frac{1}{3}\left(1 - \frac{n}{6} + \ldots\right) \tag{A6}$$

$$(B_n/B_\infty) \to (n^2/6) + \ldots \tag{A7}$$

As $n \to \infty$, $\bar{L}_n \to 0$, $B_n \to B_\infty$.

The overall behavior of the functions $\bar{L}_n/L_0$ and B_n/B_∞ is plotted in Figure A1a and Figure A1b, respectively. Equation A6 and Figure A1a show the important simple result that the average length of the first fragments released is $L_0/3$. According to eq A7 and Figure A1b, there is an initial parabolic behavior of the function B_n/B_∞, but this soon becomes linear, and then levels off.

Our goal is to calculate values of L_0 and B_∞ by curve fitting to the experimental data on average length and amount of material in fraction S2 as a function of time of digestion. There are three analyses that we have made.

(1) We assume $n = kt$, where t is the time of digestion (i.e., we assume the enzyme is acting linearly with time and attempt to fit eq A4 to the experimental data given in Figure A1a by curve fitting for different trial values of k and L_0. This analysis is shown in Figure A1a.

(2) We assume $n = kt$ and fit observed values for the amount of material released as a function of time to eq A5. This analysis, which requires curve fitting for different trial values of k and of B_∞, is shown in Figure A1b.

(3) We eliminate n (and thus k) from the analysis by plotting $\bar{L}_n/L_0$ as a function of B_n/B_∞ for a range of values of n. This curve is compared with experimental data on fragment length as a function of amount of DNA released in Figure A1c.

The model may be oversimplified for the complicated experimental situation. The experimental data are of limited accuracy. Nevertheless, the several methods of analysis all lead to estimates that L_0, the average length of a template active segment, is approximately 6500 ± 500 nucleotide pairs and that B_∞, the fraction of the genome which is template active, is 0.20 ± 0.03.

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