CHROMATIN STRUCTURE AND GENE EXPRESSION

thesis by

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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^{++}-soluble and insoluble chromatin fractions. The kinetics of production of these chromatin fractions have been investigated. After a brief enzyme treatment (5 min under standard conditions), 11% of the input chromatin DNA is found in the Mg^{++}-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 middle repetitive and nonrepetitive DNA sequences of the rat genome. Cross-reassociation experiments show that a fractionation of whole genomal DNA sequences has been achieved. Moreover, the Mg^{++}-soluble fraction of liver chromatin is enriched in nonrepeated sequences coding for liver RNA but not for brain RNA. 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. The Mg^{++}-soluble, template-active fraction is enriched five-fold in DNA sequences complementary to RNA.

The Mg^{++}-soluble fraction is enriched in nonhistone chromosomal proteins and depleted in histone protein. Histone I (f1) is absent

from the Mg^{++}-soluble active fraction. About half of the DNA of both Mg^{++}-soluble and Mg^{++}-insoluble fractions is resistant to prolonged digestion with DNAase II or staphylococcal nuclease. The nuclease-resistant structures of inactive (Mg^{++}-insoluble) chromatin are DNA-histone complexes which sediment at 11-13S. Two nuclease-resistant species are present in active chromatin. These particles sediment at 15 and 20S, respectively, and contain DNA, RNA, histone and non-histone proteins. Thermal denaturation studies suggest that the DNA of active chromatin is complexed primarily with nonhistone proteins.

TABLE OF CONTENTS

I. INTRODUCTION

The phenomena of differentiation and cellular specialization in eukaryotes can be traced, in part, to differential gene activity. This statement rests on three very important observations: first, the various cell types of a given organism possess the same genetic complement (Gurdon, 1962); second, differentiated cells of metazoan creatures transcribe only a limitted fraction of their full genetic potential (Brown and Church, 1972; Grousse et al., 1972); and, third, the RNA populations of cells are, to a certain degree, tissue-specific (Grousse et al., 1972; Ryffle and McCarthy, 1975). When compared to the transcription of protein-free DNA, the in vitro transcription of isolated chromatin is highly restricted (Huang and Bonner, 1962). Even though this observation was made over a decade ago, the specific chromosomal components responsible for the control of transcription have yet to be identified. Both RNA (Huang and Bonner, 1965; Britten and Davidson, 1969) and non-histone proteins (Paul and Gilmour, 1968; Davidson and Britten, 1971, 1973; Barrett et al., 1974) have been suggested as potential modulators of gene activity. It appears that the regulatory components, whatever they might be, remain intact in isolated chromatin; in vitro transcription of both the pea seed globulin gene and the globin genes has been found to be highly tissue-specific (Bonner et al., 1963; Gilmour and Paul, 1973; Axel et al., 1973).

One approach to the identification of the transcriptional control elements is to separate interphase chromatin into regions which are transcriptionally active and into regions which are transcriptionally

inert. We can then compare the constituents of these chromatin species. This is the approach taken herein: a method is described for the isolation of the "template-active" fraction of chromatin and the properties of this fraction have been investigated in detail.

This work was begun with the notion that the structure of transcriptionally active chromatin is markedly different from that of transcriptionally inactive regions of chromatin. There is a vast cytological and genetic literature which supports this contention. First of all, RNA synthesis appears to be restricted to the extended euchromatic regions of thymocyte nuclei; no transcription can be detected by E.M. autoradiography in the condensed heterochromatic regions of the nucleus (Littau et al., 1964). Second, during the pachytene stage of amphibian oogenesis, transcription takes place in the extended loops of the lampbrush chromosomes (Miller, 1965). Similarly, prior to transcription in polytene cells of Drosophila, the specific chromomeres to be transcribed undergo a marked structural alteration; the bands extend and become puffs (Berendes, 1973). This phenomenon of puffing is particularly well documented for the large Balbiani rings of Chironomus (Daneholt and Hosick, 1973; Grossbach, 1973).

Griffith (1970) and many others have found that the basic fiber diameter of interphase chromatin is 100 Å. Some 250 Å macrofibers are also seen; however, these macrofibers probably arise from the pairing or aggregation of two 100 Å fibers (Ris, 1969; Pooley et al., 1974).

Olins and Olins (1974) and Woodcock (1973) have observed regular spacings of chromatin particles (termed ν-bodies)in water-swollen nuclei centrifuged onto electron microscope grids. These particles are 60-80 Å in diameter and are joined by thin filaments of DNA 15 Å in diameter. At or near physiological ionic strength, the "particles-on-a-string" structure condenses into the 100 Å chromatin fiber (Griffith, 1975; Germond et al., 1975). The chromatin particles (the ν-bodies) probably reflect the ordered packaging of DNA in the chromatin fiber (Griffith, 1975).

Since the studies cited above were carried out with whole chromatin, unfractionated with respect to transcriptional activity, and since most of the chromatin in the cells of higher eukaryotes is transcriptionally inactive, the 100 Å-fiber represents the structure of inactive regions. Transcriptionally active chromatin has been visualized in the electron microscope in many organisms and by many workers. Griffith (1970) has investigated the chromatin of the protozoan Tetrahymena. The genome of this organism is similar to that of typical higher eukaryotes; both repetitious and unique sequences are found and the DNA is complexed with both histone and nonhistone proteins. This organism is dissimilar from higher eukaryotes in that 40% of the genome appears to be transcriptionally active. Electron microscope studies of Tetrahymena nuclei lysed directly on grids show fibers 30 Å in width. No 100 Å or 250 Å-fibers were observed (Griffith, 1970). Similarly, Miller (1965) has

observed a fiber diameter of about 30 Å in the active loops of lampbrush chromosomes. These loops are connected by inactive chromatin fibers 200 Å in diameter. Hamkalo (personal communication) has observed a structural transition in the chromatin of a Trypanosome. When this organism is transcriptionally dormant, the chromatin is seen in the "particles-on-a-string" or ν-body configuration. Upon induction of RNA synthesis, the chromatin fibers appear more extended with a larger and more random spacing between particles.

Other electron microscope studies of chromatin have identified several classes of chromatin fibers: Oudet et al. (1975) have fractionated chromatin into dense and light fractions in glycerol gradients. The dense fraction exhibits a regular close packing of particles (termed nucleosomes by these authors). In the light fraction, regions of close-packed particles are separated by long stretches (0.5 to >10μm) of thin fibers 15-25 Å in diameter. This light fraction could correspond to active chromatin, but this has not been proven. Langmore and Wooley (1975) have obtained qualitatively similar results with the high resolution scanning transmission electron microscope. Larger particle diameters, however, were observed by these authors when unstained and unfixed chromatin samples were examined. Griffith (1975) has demonstrated that SV 40 DNA complexed with host histones is compacted 1/4th to 1/7th its native contour length. SV 40 "chromatin" exhibits ν-body or nucleosome structures (Griffith, 1975; Germond et al., 1975). It is interesting to note that ribosomal genes in the act of transcription

are the length of their transcription product (pre-rRNA) (Miller and Hamkalo, 1972). Thus it is likely that the fully extended 15-30Å chromatin fiber corresponds to genetically active chromatin while the 100 Å or particulate fiber corresponds to inactive regions of chromatin.

There is further genetic and cytological data which demonstrates that condensed chromatin is transcriptionally inert. Perhaps the most striking example of this sort is X-inactivation (Lyon, 1961). Here the condensation of one X-chromosome leads to the total silencing of its genetic potential. Similarly, translocations of euchromatic segments to chromosomal regions proximal to condensed heterochromatin can lead to the inactivation of the euchromatic segment. Position effect phenomena of this sort are documented in both the mouse and in Drosophila (Baker, 1968). The morphological differences between active and inactive regions of chromatin as revealed by both the electron microscope and the light microscope must have a biochemical basis. Separation of chromatin into active and inactive regions should allow us to determine the chromosomal elements responsible for both structure and genetic activity.

The first attempt at chromatin fractionation was reported by Frenster, Allfrey and Mirsky (1963). Chromatin from calf thymus lymphocytes was sonicated and separated into euchromatin and heterochromatin by differential centrifugation. Based on the criteria of cofractionation of nascent RNA chains and microscopic appearance, the fractionation appeared successful. About 80% of the nuclear

DNA was found in the heterochromatin fraction, but this fraction contained only 14% of the newly synthesized RNA. The heterochromatin appeared as dense masses of 100 Å fibers; the euchromatin fraction was composed, however, of 50 Å fibrils. In a subsequent report (Frenster, 1963), the euchromatin fraction was found to have a decreased histone to DNA ratio and an increased content of nonhistone chromosomal proteins. The approach of differential centrifugation of sonicated or pressure-sheared chromatin in sucrose gradients has been taken by Chalkley and Jensen (1968), Yunis and Yasmineh (1971), Duerkson and McCarthy (1971), Nishiura (1972), Murphy et al.(1973), Warnecke et al. (1973), McCarthy et al. (1973), Chesterton et al. (1974), and most recently by Doenecke and McCarthy (1975a,b). The major findings of these studies may be summarized as follows: the light chromatin fractions (euchromatin) exhibit a high template capacity for the support of RNA synthesis with exogenous polymerase. This result has now been obtained with both prokaryotic and eukaryotic RNA polymerases. Newly synthesized RNA molecules cosediment with the light chromatin fraction; further, different protein populations are found in the light and dense chromatin fractions. In general, light chromatin is enriched in nonhistone protein and depleted in histone protein. The heterochromatin fractions are enriched in non-transcribed satellite DNA. Doenecke and McCarthy (1975a) have concluded that the basis for chromatin fractionation is differential aggregation. Although the light chromatin fraction has many of the properties expected for transcriptionally-active chromatin (high tem-

plate activity, association of nascent RNA), the crucial hybridization experiments have yet to be reported. In vivo template activity can only be established by demonstrating that the light fraction is enriched in DNA sequences which code for cellular RNA. Since the fraction of the genome which is transcribed in any given cell type is limited, the DNA of the active fraction must comprise a small subset of the genome. Furthermore, the size of this subset should correspond to the fraction of the genome which is transcriptionally active in the particular cell type under study. The validity of the sucrose gradient fractionation method can only be established once such hybridization experiments have been carried out.

McConaughy and McCarthy (1972) have fractionated pressure-sheared chromatin by thermal chromatography on hydroxyapatite. The chromatin DNA which elutes at the lowest temperatures was found to be enriched in sequences complementary to RNA from homologous but not heterologous tissues. This procedure has the major drawback that only the active DNA can be recovered from hydroxyapatite; native protein-DNA interactions are destroyed by thermal denaturation. Janowski et al. (1972) have separated pressure-sheared chromatin by gel exclusion chromatography. The exluded fractions (extended chromatin) preferentially bind ^{3}H-labeled E. coli RNA polymerase; it was also reported that the excluded fractions carry nascent RNA chains.

Another chromatographic fractionation procedure which appears highly promising is anion-exchange chromatography on ECTHAM-cellulose using

a gradient of increasing pH (Reeck et al., 1972; Simpson and Reeck, 1973). Although no differences were found in the overall protein to DNA ratios of the fractions obtained from ECTHAM-cellulose columns, the late-eluting (high pH) chromatin is enriched in nonhistone protein and depleted in histone protein. Histone I (f1) is greatly diminished in the late-eluting fractions. In agreement with the findings of McConaughy and McCarthy (1972), the late-eluting, putatively active chromatin melts at lower temperatures than the early-eluting material. The DNA of the late-eluting fraction is more available for binding RNA polymerase (Simpson, 1974) and more susceptible to nucleolytic attack (Simpson and Polacow, 1973) than the DNA of the early-eluting chromatin. Circular dichroism spectroscopy suggests that the late-eluting chromatin is in an extended DNA-like conformation (Polacow and Simpson, 1973). The ECTHAM-cellulose fractionation method appears to produce a chromatin fraction with all the properties one would expect of transcriptionally active chromatin; however, the recent report of Howk et al. (1975) demonstrates that neither the late-eluting fraction from ECTHAM-cellulose nor the light fraction from glycerol gradients are enriched in specific transcribed sequences. No differences in the distribution of transcribed viral sequences were found in the chromatin fractions obtained from either infected or noninfected cells. Further hybridization experiments are needed to determine whether the ECTHAM-cellulose method actually does separate active from inactive chromatin.

Turner and Hancock (1974) have separated sheared mouse chromatin by partition in a two-phase aqueous polymer system; the fractions obtained by this method were found to differ in their content of nonhistone proteins. Sheared chromatin has also been fractionated by equilibrium centrifugation in gradients of $CsSO_4$ (Wilt and Ekenberg, 1971), chloral hydrate (Hossainy et al., 1973), renografin (Chan and Scheffler, 1975), and metrizamide (Rickwood et al., 1974; Monahan and Hall, 1974). Although sheared chromatin can be resolved into species which differ in density, protein composition and template activity, Rickwood et al. (1974) have concluded that the metrizamide chromatin fractions do not correspond to active and inactive regions. No significant differences were found in the concentration of globin genes in the chromatin fractions obtained from either erythropoietic or nonerythropoietic cells. These results show that the criteria of chemical composition and in vitro template activity are insufficient to demonstrate that a particular chromatin fraction is truely transcriptionally active in vivo.

This laboratory has adopted an altogether different approach to the problem of chromatin fractionation. Previous attempts at fractionation have begun with sonicated or pressure-sheared chromatin. It is highly likely that these methods of shearing result in the rearrangement of chromosomal proteins. Indeed, Noll et al. (1975) have found significant differences in the subunit structure of native and mechani-

cally-sheared chromatin. To allow for the subsequent fractionation into active and inactive regions, chromatin DNA must be sheared to a size smaller than the average unit of transcription. Marushige and Bonner (1971) introduced limitted digestion of chromatin with the endonuclease DNAase II for this purpose. In our hands, nuclease digestion does not lead to detectable levels of protein rearrangement (Gottesfeld et al., unpublished). In the nucleus, nontranscribed heterochromatin is highly aggregated while transcriptionally-active euchromatin is extended (Littau et al., 1964). It is well known that isolated chromatin is insoluble at or near physiological ionic strength. Therefore, Marushige and Bonner (1971) postulated that inactive regions of chromatin should be precipitated by salt while active chromatin, which is extended in vivo, should remain soluble. It has been found that the bulk of chromatin DNA is precipitated with either saline-citrate ($[Na^+]$ = 0.2 M) or $MgCl_2$ (2 mM) while a minor fraction of DNA remains soluble (Marushige and Bonner, 1971; Billing and Bonner, 1972). Moreover, the amount of DNA in the soluble fraction varies depending upon the source of the chromatin, but corresponds quite closely to the measured template activity of the particular chromatin under study (Billing and Bonner, 1972). This thesis extends the work of Marushige and Bonner (1971) and Billing and Bonner (1972). The Mg^{++}-soluble fraction of rat-liver chromatin is enriched in sequences coding for the RNA of homologous but not heterologous tissue. Thus the Mg^{++}-soluble fraction corresponds to transcriptionally-active chromatin in

vivo. This fraction also comprises a subset of nonrepeated DNA sequences and a subset of families of repeated sequences. The Mg^{++}-soluble active fraction contains a high proportion of nonhistone chromosomal proteins and a lower histone to DNA ratio than either unfractionated chromatin or the Mg^{++}-insoluble fraction. In agreement with the findings of Reeck et al. (1972) and Simpson and Reeck (1973), histone I (f1) is absent from the active fraction.

It appears that all the DNA in chromatin is organized into regions which are sensitive to prolonged nuclease treatment and into regions which are nuclease-resistant. The nuclease-resistant structures of inactive chromatin are DNA-histone complexes; these structures resemble the ν-bodies observed by Olins and Olins (1974) (Gottesfeld et al., 1975). The nuclease-resistant structures of active chromatin are more heterogeneous than those of inactive chromatin; they are complexes of DNA, RNA, histone and nonhistone protein. Thermal denaturation studies suggest that the DNA of active chromatin is complexed primarily with the nonhistone proteins. In agreement with the results of McConaughy and McCarthy (1972), active chromatin melts at lower temperatures than inactive chromatin. Circular dichroism studies suggest that active chromatin is in a conformation more like that of protein-free DNA than that of inactive chromatin (Polacow and Simpson, 1973; Gottesfeld et al., herein).

The discovery of single copy and repeated sequences in eukaryotic DNA (Britten and Kohne, 1968) suggested that repetitious sequences might be control elements in the modulation of transcription (Britten and Davidson, 1969; Davidson and Britten, 1973). Supporting evidence for this model comes from the finding of general interspersion of moderately repetitive and single copy sequences throughout the genome (Davidson and Britten, 1973; Davidson et al., 1973; Graham et al., 1974; Davidson et al., 1975a) and the finding that polysomal mRNA is transcribed mainly from the nonrepeated fraction of the genome (Davidson and Britten, 1973; Lewin, 1975). Furthermore, sequences which are transcribed into mRNA are found adjacent to repetitive sequences in the DNA of the sea urchin (Davidson et al., 1975b). It is tempting to speculate that the binding of activator molecules to middle repetitive sequences promotes the transcription of neighboring single copy structural gene sequences. In its simplest form, the Britten-Davidson model predicts that if a given repetitive sequence is adjacent to a transcribed single copy sequence, then all the members of this repetitive family are also adjacent to transcribed sequences. Thus we would predict that only a limited fraction of the repetitive complexity of the genome should be found adjacent to transcribed single copy sequences. The results presented herein are consistant with this view. The active fraction of rat-liver chromatin (which is enriched five-fold in transcribed single copy sequences) contains about 11% of the total single copy complexity and 5-8% of the repeti-

tive complexity of the rat genome. This demonstrates that the distribution of repetitive sequences in rat DNA is decidedly nonrandom. What is more, preliminary results suggest that repetitive and single copy sequences are interspersed in the active fraction. These results offer insights into the functional organization of the eukaryotic chromosome; hopefully in the near future we will have a clear picture of the workings of the eukaryotic gene regulatory apparatus.

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II. ISOLATION OF TEMPLATE ACTIVE AND INACTIVE REGIONS OF CHROMATIN

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[8] Isolation of Template Active and Inactive Regions of Chromatin

By JAMES BONNER, JOEL GOTTESFELD, WILLIAM GARRARD, RONALD BILLING, and LYNDA UPHOUSE

In the differentiated eukaryotic cell, only a portion of the nuclear DNA is transcribed into RNA while the majority of the genetic material is repressed. Part of this transcriptional heterogeneity appears to be determined by the protein complement of chromosomes. To better understand the role of chromosomal proteins in chromatin function and structure, we and other investigators have developed techniques to fractionate chromatin into template active and inactive regions. Such an approach encounters two main technical problems. First, chromatin must be sheared to a size small enough to allow the subsequent separation of active and inactive regions. Second, a means of selectivity fractionating the material is required.

To minimize cross-contamination of active and inactive regions, chromatin should be sheared to less than the size of an average unit of transcription. Most investigators have used mechanical methods (including sonication[1] and French pressure cell passage[2,3]) to shear chromatin. Marushige and Bonner[4] have introduced the use of DNase II for this purpose. Nucleolytic cleavage of chromatin DNA is gentle and does not lead to detectable levels of protein rearrangement during shearing or fractionation. We have adopted this technique and describe appropriate conditions on p. 98.

Sheared chromatin has been separated into active and inactive regions

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[3] M. Janowski, D. S. Nasser, and B. J. McCarthy, *in* "Karolinska Symposia on Research Methods in Reproductive Endocrinology" (E. Diczfalusy, ed.), 5th Symposium, p. 112. Karolinska Institutet, Stockholm, 1972.

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by differential centrifugation,[1,5] and by chromatography on hydroxyapatite,[2] agarose,[3] and anion-exchange resin.[6] Marushige and Bonner[4] have developed a simple and rapid fractionation technique which involves selective precipitation of the inactive region with standard saline-citrate. This approach is based on the fact that chromatin is highly aggregated at physiological ionic strength. Under such conditions, the limited portions of chromatin available to RNA polymerase would be predicted to be less aggregated and perhaps soluble in sheared chromatin preparations. Our method, based on the same principle, uses $MgCl_2$ to precipitate the inactive region. The minor portion of chromatin soluble in 2.0 m*M* $MgCl_2$ is the template active region.

Procedure for Fractionation

Sucrose-purified chromatin[7] is homogenized at 4° in 10 volumes of 10 m*M* Tris·Cl (pH 8) using a glass-Teflon homogenizer (10 strokes by hand followed by 2 minutes of gentle motor homogenization). Unless otherwise stated, all subsequent operations are performed at 4°. The resulting chromatin solution is dialyzed overnight against 200 volumes of 25 m*M* sodium acetate (pH 6.6) and pelleted by centrifugation at 25,000 *g* for 20 minutes. The resulting material is homogenized as above in 25 m*M* sodium acetate (pH 6.6) to yield a chromatin solution having an $A_{260}^{1\ cm}$ of 10 (measured in 1 *N* NaOH). The solution is brought to 24° and DNase II (Worthington, HDAC) is added to a final concentration of 100 units/ml (10 units of enzyme per A_{260} unit of chromatin). After incubation at 24°, the pH of the solution is adjusted to 7.5 by the addition of 0.1 *M* Tris·Cl (pH 11) and the mixture is cooled to 4°. Unsheared chromatin is removed by centrifugation at 25,000 *g* for 20 minutes. The amount of chromatin DNA remaining in the supernatant is naturally dependent upon the duration of incubation with DNase. After 5 minutes of enzyme treatment, 15% of the chromatin DNA is found in the supernatant, while after prolonged incubation (15 minutes or more), 70 to 85% is found.

The sheared chromatin is now fractionated into template active and inactive portions by $MgCl_2$ precipitation. One ninety-ninth volume of 0.20 *M* $MgCl_2$ is added dropwise with rapid stirring. After 30 minutes of further stirring, the turbid suspension is centrifuged at 25,000 *g* for

[5] G. R. Chalkley and R. Jensen, *Biochemistry* **7**, 4380 (1968).

[6] G. R. Reeck, R. T. Simpson, and H. A. Sober, *Proc. Nat. Acad. Sci. U.S.* **69**, 2317 (1972).

[7] J. Bonner, G. R. Chalkley, M. Dahmus, D. Fambrough, G. Fujimura, R.-C. Huang, J. Huberman, R. Jensen, K. Marushige, H. Ohlenbusch, B. Olivera, and J. Widholm, this series, Vol. 12B, p. 3.

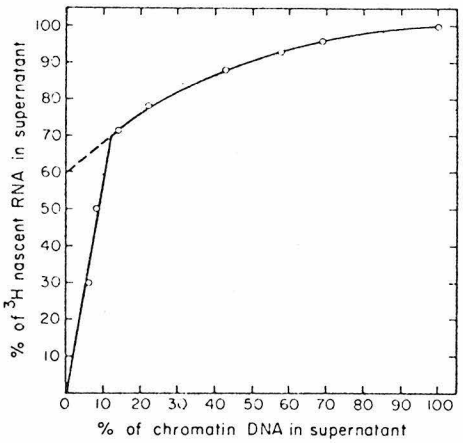

FIG. 1. Localization of ^{3}H-labeled nascent RNA. Sheared chromatin was prepared from ascites cells, pulse labeled for 5 minutes with [^{3}H]uridine as described by R. J. Billing and J. Bonner [*Biochim. Biophys. Acta* 28, 453 (1972)]. Aliquots of chromatin were precipitated with various concentrations of $MgCl_2$. Soluble DNA and radioactivity were determined. Circles along curve represent successively decreasing $MgCl_2$ concentrations used for precipitation of template inactive portions.

20 minutes. The resulting supernatant contains the template active chromatin fraction, while the pellet contains the template inactive material. The length of the DNA in the template active fraction is dependent upon the conditions of nuclease treatment. A 5 minute exposure produces fragments of 700–850 base pairs (500 nucleotides single stranded).[8] Longer times of incubation produce much shorter fragments.[9]

Evidence for Fractionation

Evidence for the separation of the template active and inactive regions by $MgCl_2$ precipitation is based on the cofractionation of nascent RNA with the active region.[9,10] Chromatin was prepared from Novikoff ascites cells[11] which were pulse labeled for 5 minutes with [^{3}H]uridine. Under such conditions, the majority of the label would be predicted to be associated with the template active region as growing RNA chains. The chromatin was then sheared with DNase II, and aliquots were precipitated with decreasing concentrations of $MgCl_2$. Figure 1 shows the

[8] The size of the sheared DNA was estimated by neutral and alkaline sedimentation [W. F. Studier, *J. Mol. Biol.* **11**, 373 (1964)].

[9] R. J. Billing and J. Bonner, *Biochim. Biophys. Acta* 28, 453 (1972).

[10] J. Bonner, W. T. Garrard, J. Gottesfeld, D. S. Holmes, J. S. Sevall, and M. Wilkes, *Cold Spring Harbor Symp. Quant. Biol.* 38, 303 (1973).

[11] M. E. Dahmus and D. J. McConnell, *Biochemistry* 8, 1524 (1969).

CHEMICAL COMPOSITION OF THE TEMPLATE ACTIVE AND INACTIVE REGIONS OF CHROMATIN[a]

	Novikoff ascites[b]			Rat liver[c]		
Sample	% Total DNA	Acid-extractable protein: DNA	Nonhistone: DNA	% Total DNA	Acid-extractable protein: DNA	Non-histone: DNA
Sheared chromatin	80 ± 6	1.14 ± 0.02	0.55 ± 0.20	78 ± 3	1.12	0.36
Template active	12 ± 1	0.43 ± 0.19	0.85 ± 0.20	24 ± 2	0.60	0.46
Template inactive	67 ± 6	1.24 ± 0.04	0.50 ± 0.15	53 ± 4	1.43	0.29

[a] Chemical composition was determined as follows. DNA was estimated by absorbance at 260 nm in 1 *N* NaOH (1.0 A_{260} = 34 μg/ml). Chromatin fractions were extracted twice with 0.4 *N* H_2SO_4. The nonhistone residue was dissolved in 1 *N* NaOH. Protein was estimated by a modification of the method of H. Kuno and H. K. Kihara [*Nature (London)* **215**, 974 (1967)], using 25% TCA for precipitation. Purified histone and bovine serum albumin were used as standards.

[b] Average of five preparations plus or minus standard deviation.

[c] For DNA, average of three preparations plus or minus standard deviation. For protein, average of two preparations. Chromatin from both rat liver and ascites cells was incubated with DNase II for 15 minutes prior to $MgCl_2$ fractionation.

amounts of chromatin DNA and nascent RNA remaining soluble at various $MgCl_2$ concentrations. The bulk of labeled RNA remains in solution with a small portion of the DNA until the $MgCl_2$ concentration becomes sufficiently high to precipitate the active region. From the initial slope of the fractionation curve, it is estimated that the active region has been enriched 5.5-fold over unfractionated chromatin. (This value is estimated by assuming that all the labeled RNA is associated with the template active region. The enrichment of template active DNA would be greater if any redistribution of the labeled RNA had occurred.) More direct evidence for fractionation is based on DNA renaturation, RNA–DNA hybridization, and template activity measurements, and is reported elsewhere.[12]

Properties of the Chromatin Fractions

The table shows the chemical compositions of chromatin fractions isolated from Novikoff ascites and rat liver. The template active regions

[12] J. M. Gottesfeld, W. T. Garrard, G. Bagi, R. F. Wilson, and J. Bonner, *Proc. Nat. Acad. Sci. U.S.* **71**, 2193 (1974).

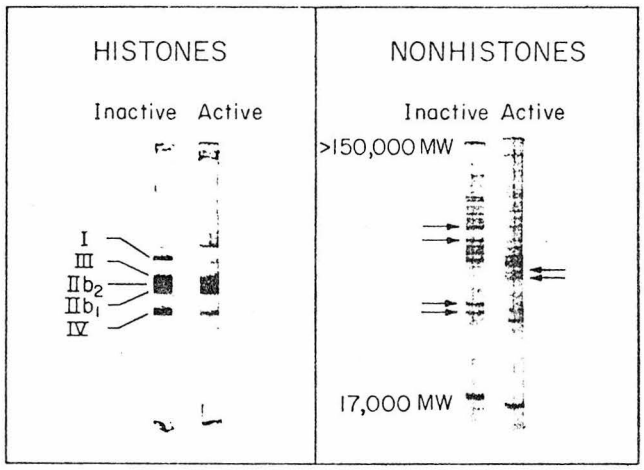

FIG. 2. Disc electrophoresis of chromosomal proteins from the template active and inactive regions. Histone gels: Electrophoresis and staining was performed as described by S. Panyim and G. R. Chalkley [*Arch. Biochem. Biophys.* 130, 337 (1969)]. Each gel was loaded with 30 μg of acid-extracted, ethanol-purified protein. Nonhistone gels: Electrophoresis and staining was performed as described by J. King and U. K. Laemmli [*J. Mol. Biol.* 62, 465 (1972)]. Acid-extracted chromatin was homogenized in and dialyzed against 2.5% sodium dodecyl sulfate, 65 m*M* Tris · Cl (pH 6.8). Prior to electrophoresis, metastable aggregates were disrupted by adding 5% β-mercaptoethanol followed by boiling for 1 minute. Gels were each loaded with 100 μg of protein.

from both sources are impoverished in acid-extractable protein, while the template inactive fractions are enriched in this material. Fractionation of chromatin also yields an unequal partitioning of the nonhistone proteins. The active region contains about twice the amount of nonhistone protein per milligram of DNA as the inactive region. About 80% of the chromatin of both ascites and liver is sheared by prolonged (15 minute) enzyme treatment. The amount of template active DNA found in the supernatant after $MgCl_2$ fractionation is strikingly different, however, in the two cases. The proportion of rat liver template active DNA is 2-fold higher than that of ascites, 24% versus 12%. These values are in accordance with the previously reported template activities of the two chromatins as measured with exogenous RNA polymerase.[13]

Disc electrophoretic patterns of the acid-extractable proteins from the active and inactive fractions of rat liver chromatin are shown in Fig. 2. The most pronounced difference in the banding patterns is the complete absence of histone I in the active region. Furthermore, although over 95%

[13] J. Bonner, M. E. Dahmus, D. Fambrough, R.-C. Huang, K. Marushige, and D. Y. H. Tuan, *Science* 159, 47 (1968).

of the acid-extractable protein from sheared chromatin and inactive region fractions are histone proteins, we estimate that only 60% of the acid-extractable protein from the active region is histone (from the amount of protein that is applied to the gels and the intensity of the histone staining bands). On this basis the true histone:DNA ratio of the template active fraction is less than 0.36, while the nonhistone:DNA ratio of this fraction is greater than 0.70. It is clear that the nonhistone proteins are concentrated in the template active fraction and impoverished in the template inactive fraction. Separation of nonhistone polypeptides on the basis of molecular size by sodium dodecyl sulfate disc electrophoresis is also shown in Fig. 2. The inactive region has a higher proportion of high molecular weight bands. In addition, quantitative differences in the concentration of individual bands exist (marked with arrows).

Comment

Sheared chromatin can be fractionated into template active and inactive portions on the basis of predicted differences in the physical properties between the two regions. If separation without gross cross contamination is to be achieved, the size of the fragments is critical. Mild digestion with DNase II has the obvious advantage over other reported techniques in that it yields substantially smaller fragments. Fractionation of chromatin with $MgCl_2$ provides a rapid and reliable means of separating active and inactive regions.

Acknowledgments

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III. PARTIAL PURIFICATION OF THE TEMPLATE-ACTIVE FRACTION OF CHROMATIN: A PRELIMINARY REPORT

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Partial Purification of the Template-Active Fraction of Chromatin: A Preliminary Report

(chromatin fractionation/DNase II/DNA·RNA hybridization)

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Contributed by James Bonner, March 18, 1974

ABSTRACT **A fraction of rat-liver chromatin that is transcriptionally active *in vivo* has been purified 6- to 7-fold over whole chromatin. This was accomplished by selectively shearing chromatin with DNase II followed by fractionating the released portion on the basis of its solubility properties in 2 mM $MgCl_2$. The resulting soluble material comprises 11% of the total chromatin DNA and is impoverished in histone and enriched in nonhistone protein. Compared with unsheared chromatin, this minor fraction exhibits marked differences in chromosomal protein species. DNA renaturation studies indicate that this fraction is composed of a specific subset of whole genomal DNA sequences. Furthermore, DNA·RNA hybridization experiments suggest that almost 60% of the nonrepetitious DNA sequences of this minor fraction could code for cellular RNA.**

Differentiated eukaryotic cells transcribe a limited and tissue-specific portion of their nuclear DNA sequences (1, 2). It is now well established that transcription is restricted in isolated chromatin (3). Furthermore, recent evidence suggests that at least some of the mechanisms of genetic regulation remain intact in isolated chromatin (4, 5). At present, however, no conclusive data are available to indicate which components of chromatin serve as specific regulators of genetic activity. A direct approach to this question would be to study the components of the minor portion of chromatin that is transcriptionally active. This, however, requires as a prerequisite the development of chromatin fractionation techniques.

Two principal problems are encountered in designing a strategy for the fractionation of chromatin. First, to avoid cross-contamination, chromatin must be sheared to a size less than that of the average unit of transcription. Ideally, this should be accomplished by a method that does not lead to protein denaturation, rearrangement, or dissociation. Second, a gentle method of physically separating "active" from "inactive" material is required. Several groups have introduced fractionation techniques based on mechanical shearing followed by separation by differential centrifugation or column chromatography (6–10). An alternative method suggested by workers in our laboratory utilizes DNase II for shearing, and selective precipitation by mono- or divalent cations for chromatin fractionation (11, 12).

Previous studies have shown that DNase II preferentially attacks a minor portion of chromatin DNA; the amount of this "DNase-labile" fraction varies depending on the chromatin source but corresponds directly to the ability of the given chromatin to serve as a template for exogenous RNA polymerase (13). Furthermore, fractionation experiments using chromatin prepared from hepatoma cells pulse-labeled with [^{3}H]uridine reveal that over 60% of the label fractionates with 10% of the chromatin DNA (13). These observations prompted us to examine this technique of chromatin fractionation in more detail. In the present communication we report that rat-liver chromatin has been fractionated into a predominantly "active" component that differs in at least five ways from whole chromatin: chemical composition, chromosomal protein populations, template activity for support of RNA synthesis, DNA sequence complexity, and DNA sequence homology with cellular RNA.

Abbreviation: C_0t, molar concentration of DNA nucleotides multiplied by time of incubation.

METHODS

Chromatin Fractionation. Rat-liver chromatin purified by sucrose gradient centrifugation (3) was washed once with 10 mM Tris·HCl (pH 8) and dialyzed overnight at 4° against 200 volumes of 25 mM sodium acetate buffer (pH 6.6). The volume of the dialysate was adjusted to give an $A_{260\,nm}^{1\,cm}$ of 10 (measured in 0.9 N NaOH). The solution was brought to 24°, and DNase II (Worthington, HDAC) was added to 100 units/ml. The reaction was terminated after 5-min incubation by the addition of 50 mM Tris·HCl (pH 11) to pH 7.5 and cooling on ice. Unsheared chromatin (P1) was removed by centrifugation at 27,500 × *g* for 20 min at 4°. To the supernatant one ninety-ninth volume of 0.2 M $MgCl_2$ was added dropwise with stirring at 4°. After 30 min of additional stirring, the suspension was centrifuged as above yielding a pellet (P2) and supernatant (S2) fraction.

Chromatin Protein Analyses. Histone and nonhistone protein were determined as described (3). Acid-extracted protein was purified by ethanol precipitation and analyzed by disc electrophoresis as reported elsewhere (14). The acid-insoluble chromatin residue was homogenized in and dialyzed against 2.5% (w/v) sodium dodecyl sulfate–65 mM Tris·HCl (pH 6.8)–2% 2-mercaptoethanol. Samples were heated for 1 min before electrophoresis (15). Gels were scanned at 600 nm with a Gilford 2000 spectrophotometer.

DNA Reassociation Kinetics. DNA was purified from various chromatin fractions as described elsewhere (16). DNA isolated from the S2 chromatin fraction had a single-stranded length of 500 nucleotides, as determined by sedimentation velocity centrifugation under alkaline conditions (17). DNA isolated from total chromatin and from the P1 chromatin fraction was sheared by two passes through a Ribi-Sorvall

TABLE 1. *Properties of chromatin fractions*

Chromatin sample	% Chromatin DNA*	Template activity†	Composition relative to DNA (w/w) Histone protein‡	Composition relative to DNA (w/w) Nonhistone protein
Unfractionated Fraction	100	20	1.06	0.65
P1	84.6 ± 4.8	9	1.15	0.58
S2	11.3 ± 3.9	65	0.61	1.60
P2	4.1 ± 2.5	—§	—	—

* Mean of 11 determinations ± SD as estimated by absorbance at 260 nm.

† Percent template activity (24) as compared to DNA isolated from the same chromatin sample. *Escherichia coli* RNA polymerase (fraction IV) was prepared according to McConnell and Bonner (25).

‡ Histone protein determined from areas of densitometer scans of polyacrylamide gels (14) loaded with known amounts of proteins.

§ Not determined.

pressure cell at 50,000 lbs/inch². This procedure yields double-stranded fragments 400 to 450 nucleotides in length, as measured by electron microscopy (18). Kinetics of DNA reassociation were monitored by hydroxyapatite chromatography using standard techniques (19). Data obtained at various DNA and sodium ion concentrations were normalized to C_0t values equivalent to those obtained in 0.12 M phosphate buffer (0.18 M Na^+, see ref. 19). Computer analysis was performed according to Britten *et al.* (19).

Isolation and Labeling of Nonrepetitive DNA. DNA was incubated to an equivalent C_0t of 2.5×10^2 (S2 DNA) or 1.5×10^3 (P1 DNA), and the single-stranded fraction was isolated by hydroxyapatite chromatography. This material was dialyzed against distilled water, concentrated by lyophilization, dissolved in phosphate buffer, and allowed to renature as before. Finally, the DNA that remained single-stranded after two cycles of purification was incubated to a C_0t of 10^4. The resulting duplex material was dialyzed against distilled water and then concentrated. The purified nonrepetitive DNA was labeled with ^{125}I by the Commerford method (20) as modified in our laboratory (21). Specific activities of 1×10^6 cpm/μg were obtained.

DNA·RNA Hybridization. Total cell RNA from rat liver was isolated by a modified hot phenol–sodium dodecyl sulfate extraction procedure (22) and sheared by two passes through the Ribi-Sorvall pressure cell at 30,000 lbs./inch². The resulting RNA had an average length of 1000 nucleotides, as judged by sedimentation velocity centrifugation under nondenaturing conditions (23). Hybridization experiments were performed at 74° in 30 mM sodium phosphate buffer (pH 6.5)–0.675 M NaCl–1 mM EDTA at an RNA:^{125}I-labeled DNA mass ratio of 2 to 2.5×10^4:1 and an RNA concentration of 20 mg/ml. Samples were incubated in sealed capillary tubes. Reactions were terminated by a 20-fold dilution in reaction buffer at 60° followed by application to hydroxyapatite columns equilibrated with 30 mM phosphate buffer–0.1 M NaCl at 60°. Under these conditions both single- and double-stranded nucleic acids are absorbed while free ^{125}I passes through. Single-stranded DNA and the majority of the RNA were eluted with 0.12 M phosphate buffer; DNA·RNA duplexes were eluted with 0.48 M phosphate buffer. About 20% of the sheared RNA also eluted in 0.48 M phosphate buffer. The single-stranded and hybrid fractions were precipitated with 10% trichloroacetic acid after addition of 40 μg of bovine-serum albumin per sample. The amount of DNA in DNA·RNA hybrids was determined by collecting the resulting precipitates on membrane filters, and counting dried filters in a toluene-based scintillant. At zero time of incubation, about 2% of the ^{125}I-labeled DNA eluted from hydroxyapatite in the 0.48 M phosphate buffer fraction; this background value was subtracted from all time points. DNA·DNA reassociation, estimated by incubation of ^{125}I-labeled DNA with NaOH-hydrolyzed RNA, was not detectable above zero-time binding.

RESULTS

Fractionation of Chromatin Components. The experiments described herein have utilized a standard set of conditions for chromatin fractionation (see *Methods* for details). Chromatin is first selectively sheared by incubation with DNase II for 5 min. Unsheared chromatin is removed by centrifugation, yielding a pellet termed P1. This fraction comprises 85% of the input DNA (Table 1). The resulting supernatant (S1) is fractionated on the basis of its solubility in 2 mM $MgCl_2$ into a second supernatant fraction (S2) and a minor insoluble fraction (P2). The second supernatant fraction (S2) comprises 11% of the input DNA. Fraction P1 has a chemical composition not unlike that of unfractionated chromatin (Table 1). In contrast, fraction S2 shows a great enrichment in nonhistone protein and a depletion in histones. Fraction P2 comprises only a trace of material under the present conditions of fractionation; earlier studies using conditions resulting in more extensive shearing have shown that fraction P2 consists of DNA complexed stoichiometrically with histone (13). Template activity assays with exogenous polymerase reveal that fraction S2 chromatin is 3-fold superior to unfractionated chromatin as template for RNA synthesis (Table 1). In contrast, the template activity of fraction P1 is less than that of whole chromatin.

Fig. 1 shows the disc electrophoretic profiles of the chromosomal proteins of fractions P1 and S2. The populations of histone and nonhistone proteins of fraction P1 are similar to those of unfractionated chromatin (data not shown), as one might predict, since the majority of chromosomal protein (about 85%) remains in this fraction. In contrast, the proteins of fraction S2 are quite different. Histone I is absent and histone IV is present in a reduced proportion (Fig. 1*A*). A protein band migrating slightly slower than histone I appears to be enriched in fraction S2. The nature of this component is unknown, although a band at a similar position in whole rat-liver histone preparations has been shown to have a turnover rate at least 10-fold greater than histone I protein (26). Nonhistone polypeptides of fraction S2 show striking qualitative and quantitative differences as compared to those of fraction P1 (Fig. 1*B*). It is of interest that fraction S2 is rich in two polypeptides in the molecular weight range of 38,000, the approximate size of the subunits involved in the packaging of heterogeneous nuclear RNA (27).

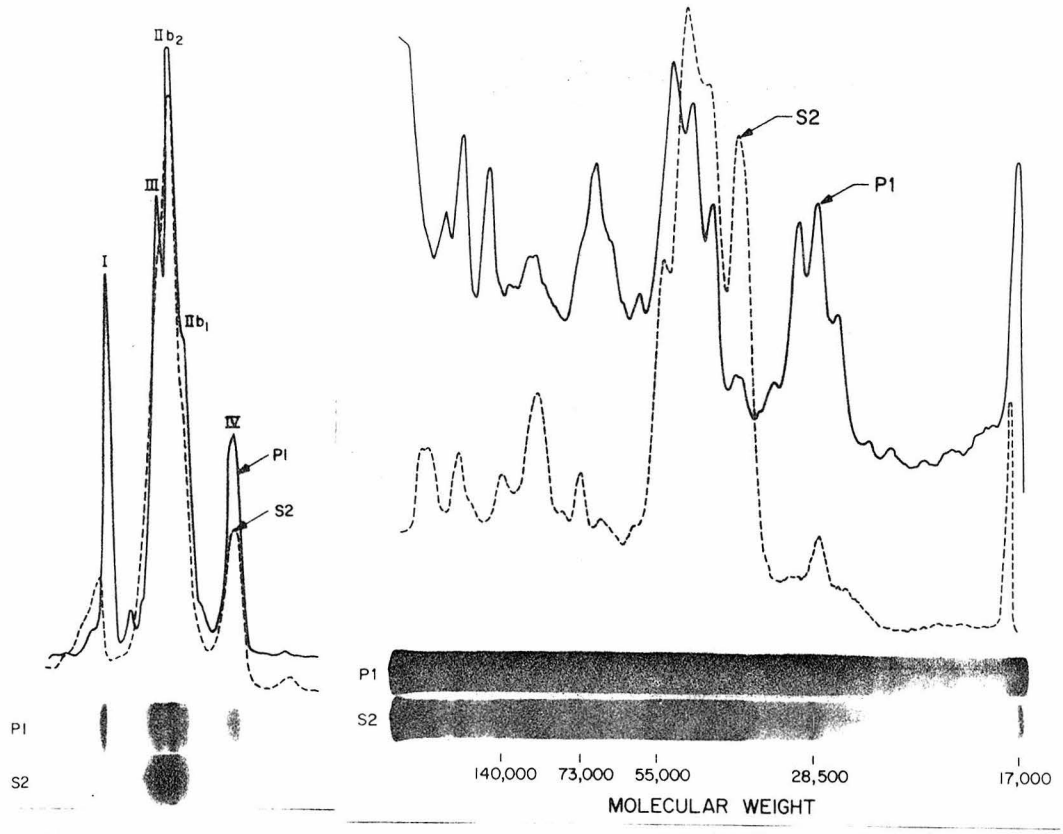

FIG. 1. Disc electrophoretic profiles of the proteins of chromatin fractions P1 and S2. (*A*) Histone protein. Acid-soluble protein (50 μg and 25 μg) from chromatin fractions S2 and P1, respectively, were separated by urea–disc gel electrophoresis. (*B*) Nonhistone chromosomal proteins. Nonhistone protein (100 μg) of each fraction was separated by sodium dodecyl sulfate–disc gel electrophoresis.

DNA Renaturation Kinetics. The kinetics of reassociation of unfractionated chromatin DNA and of fraction S2 DNA are presented in Fig. 2. Both samples have rapidly, intermediately, and slowly renaturing kinetic components, representing approximately 10%, 20%, and 70% of the input DNA, respectively. For unfractionated DNA these components correspond to highly repetitive, moderately repetitive, and nonrepetitive sequences, and agree both in amount and kinetic complexity with published data (21). In contrast, the rate of renaturation of fraction S2 DNA is strikingly different in that both the intermediately and slowly reannealing kinetic components have significantly lower $C_0t_{1/2}$ values than those of whole genomal DNA. If fraction S2 DNA were derived from a random population of chromatin DNA sequences, its reassociation curve would be identical to that of unfractionated DNA. The fact that both the intermediately and slowly reannealing components of fraction S2 DNA reassociate faster than those of unfractionated DNA clearly indicates that this material contains a specific subset of the sequences of the rat genome. The reassociation curve for fraction P1 DNA is nearly identical to that for unfractionated DNA (data not shown); this is reasonable since fraction P1 comprises 85% of the chromatin DNA.

It can be shown both by calculation and by experimentation that the slowly reannealing kinetic component of fraction S2 DNA corresponds to nonrepetitive DNA and not to some repetitive component. Fraction S2 contains 11.3% of the total chromatin DNA (Table 1), and its slow kinetic component

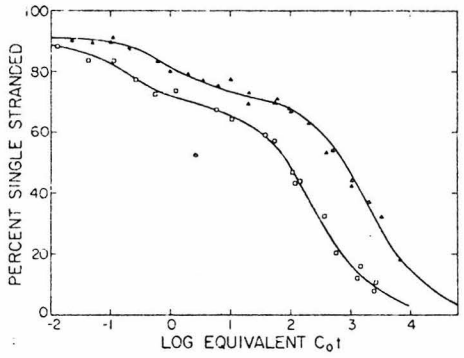

FIG. 2. Reassociation profiles of unfractionated chromatin DNA and fraction S2 DNA. Renaturation of total chromatin DNA (▲) and fraction S2 DNA (□) was assayed by chromatography on hydroxyapatite (19). The chromatin DNA points fall on the computer fit line (*solid line*) of the data of Holmes and Bonner (21). The line through the data for fraction S2 DNA was obtained by a similar computer analysis.

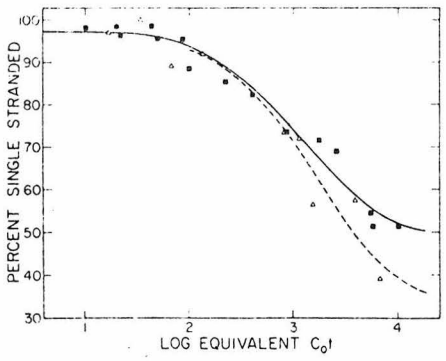

FIG. 3. Reassociation profiles of isolated nonrepetitive ^{125}I-labeled DNA in the presence of an excess of unfractionated DNA. Labeled nonrepetitive DNA (see *Methods*) of fraction S2 (■) or fraction P1 (Δ) was mixed with unlabeled total chromatin DNA in 30 mM phosphate buffer–0.675 M NaCl–1 mM EDTA, denatured for 5 min at 100°, and allowed to renature at 74°. The fraction of DNA in duplex was assayed by hydroxyapatite chromatography as described in *Methods* (section on RNA·DNA hybridization). The scale on the *abscissa*, equivalent C_0t, refers to the concentration of unlabeled DNA. Data were obtained at various concentrations of unlabeled DNA; however, the mass ratio of unlabeled DNA to labeled nonrepetitive DNA was maintained at 2 to 5 × 10^2:1. Computer analysis was used to fit lines to the data (19).

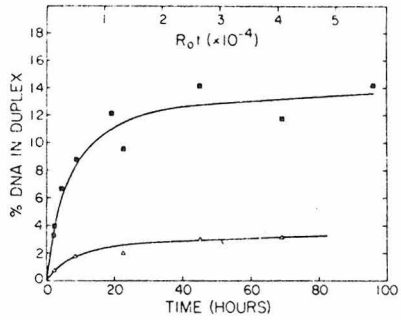

FIG. 4. Hybridization of nonrepetitive ^{125}I-labeled DNA from fractions P1 and S2 to sheared liver RNA. The mass ratio of RNA:DNA for fraction S2 DNA (■) was 2.5 × 10^4:1, while that for fraction P1 DNA (Δ) was 2.0 × 10^4:1. The *upper scale* on the *abscissa*, R_0t, is the product of time of incubation (in seconds) and molar concentration of RNA (59 mM).

comprises 63% of this fraction (Fig. 2). Since the rat haploid genome contains 1.8 × 10^{12} daltons of DNA (28), the analytical complexity of the slow component of fraction S2 DNA is (1.8 × 10^{12}) (0.113) (0.63) = 1.29 × 10^{11} daltons. The observed kinetic complexity of this component is 1.25 × 10^{11} daltons (Fig. 2; relative to *Escherichia coli*, $C_0t_{1/2}$ = 4.1). Thus, each sequence is represented approximately once in this kinetic portion of fraction S2 DNA. That this is indeed the case has been demonstrated directly by isolating this component (see *Methods*), labeling it *in vitro* with ^{125}I, and reannealing it in the presence of a vast excess of unfractionated chromatin DNA (Fig. 3). The $C_0t_{1/2}$ observed for such labeled S2 DNA was 1.3 × 10^3. Similarly, the $C_0t_{1/2}$ observed for the isolated nonrepetitive component of fraction P1 DNA was 1.5 × 10^3 (Fig. 3). The moderately repetitive sequences of fraction S2 DNA also represent a subset of the repetitive sequences of the genome. This matter will be discussed in detail elsewhere (Gottesfeld *et al.*, in preparation). At present it is not clear whether the fast reassociating component of fraction S2 DNA is analogous to highly repetitive DNA sequences, or whether it represents DNA fragments containing internal complementary sequences.

DNA·RNA Hybridization. The isolated nonrepetitive ^{125}I-labeled DNA of Fig. 3 was also hybridized to sheared, total liver RNA under conditions of vast RNA excess (Fig. 4). The saturation values, estimated from double-reciprocal plots of the data, were 3.5% and 14.5% for fraction P1 and S2 nonrepetitive DNA, respectively. In a similar experiment, 12.5% of fraction S2 nonrepetitive DNA hybridized to unsheared total liver RNA. The value of 3.5% obtained for fraction P1 is in accord with published values for the extent of transcription of nonrepetitive sequences in mouse-liver tissue (1, 2). These data suggest that fraction S2 DNA is enriched approximately 4-fold in sequences that code for cellular RNA. We consider this value to be an underestimate, since only 50% of the fraction S2 DNA used in these experiments was able to renature to whole genomal DNA at high C_0t values, while about 70% of fraction P1 DNA could form duplexes (Fig. 3). The reasons for this are unknown, but may be due to breakdown during handling and long incubations. Thus, we consider the true RNA hybridization saturation figure for fraction S2 nonrepetitive DNA to be almost 30% (or 60% if one assumes asymmetric transcription). This would represent a 6- to 7-fold enrichment in template active sequences in fraction S2 DNA over those in fraction P1 DNA. Furthermore, the data of Fig. 2 suggest that the nonrepetitive sequences of fraction S2 have been enriched by a similar factor [($C_0t_{1/2}$ total single copy DNA) ($C_0t_{1/2}$ S2 single copy DNA)$^{-1}$] = (1500) $(225)^{-1}$ = 6.7], in agreement with this prediction.

DISCUSSION

Based on several criteria, the technique we have adopted for chromatin fractionation appears to be successful. First of all, the sequences of DNA found in fraction S2 consist of a specific subset of the total genomal sequences. To the best of our knowledge, this is the first demonstration of such sequence fractionation by renaturation kinetics. Second, the nonrepetitive sequences of fraction S2 DNA are enriched in those which code for cellular RNA. This finding provides firm evidence for the partial purification of "template active" chromatin, originally suggested by experiments on the cofractionation of nascent RNA (13). Finally, chromosomal proteins have been fractionated both in a quantitative and qualitative sense.

Our success in the isolation of "template active" chromatin can be estimated in at least two ways: from saturation values obtained by DNA·RNA hybridization experiments and from assay of template activity *in vitro*. Both measurements yield values of 50–65% purity of "template active" chromatin in fraction S2. DNA reassociation studies on fraction S2 show a 6- to 7-fold enrichment of single-copy sequences over those of whole chromatin. Since 50–65% of these sequences are presumably active in RNA synthesis, isolation of pure template active chromatin would require a 9- to 14-fold purification over whole chromatin. This suggests that some 7–11% of whole genomal DNA is transcriptionally active. This estimate is in accord with published RNA hybridization data (2) for

the extent of genetic activity in liver if one assumes asymmetric transcription (3.5–5.5%). It should be noted that the contamination of fraction S2 DNA with transcriptionally inert sequences is not a random process, for if it were, the reassociation profile of fraction S2 DNA would not follow simple second-order kinetics as shown in Fig. 2.

Our findings suggest wide opportunities for the application of this technique in the study of the control of gene expression. One is the enrichment (by almost one order of magnitude) from whole chromatin of those nonrepetitive DNA sequences that are expressed in that chromatin. A second is the direct comparison of DNA sequence expression in the chromatin of different tissues, organs, or developmental states. A third might be in the study of the fidelity of reconstitution of chromatin from its several constituents.

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IV. SEQUENCE COMPOSITION OF THE TEMPLATE-ACTIVE FRACTION OF RAT-LIVER CHROMATIN

To be submitted for publication.

Summary

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^{++}-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 per A_{260} unit of chromatin at pH 6.6), 11% of the input chromatin DNA is found in the Mg^{++}-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 middle repetitive and nonrepetitive DNA sequences of the rat genome. Cross-reassociation experiments between DNA from the different chromatin fractions show that a fractionation of whole genomal DNA sequences has been achieved. Moreover, the Mg^{++}-soluble fraction of liver chromatin is enriched in nonrepeated sequences coding for liver RNA but not for brain RNA. Cross-reassociation experiments confirm that the Mg^{++}-soluble fraction of liver chromatin contains a low proportion of sequences in common with the Mg^{++}-soluble fraction of brain chromatin. Thus, fractionation does not depend merely on some general property of chromatin but is specific with regard to the template activity of the tissue from which the chromatin was obtained.

Introduction

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 (histone and nonhistone proteins), 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). It has nascent RNA associated with it (Billing and Bonner, 1972; Bonner et al., 1975), and it is

in a conformation similar to extended, native DNA in solution (Gottesfeld et al., 1974b). Moreover, this fraction is enriched in nonrepetitive sequences complementary to cellular RNA (Gottesfeld et al., 1974a).

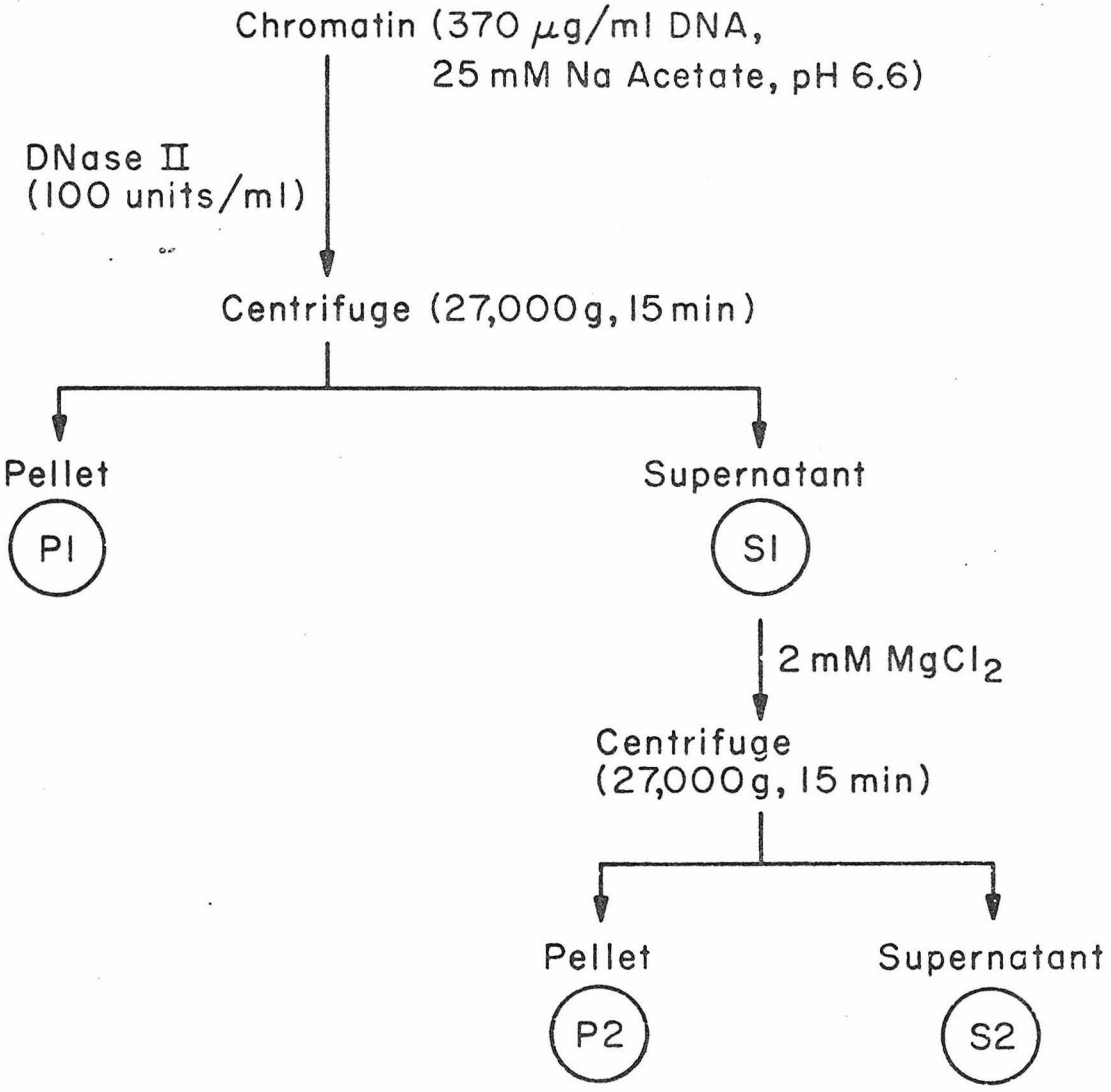
In this report, we expand upon our earlier investigations. We find that the nuclease-sensitive fraction is enriched in sequences that code for the RNA of the tissue from which the chromatin was obtained, but not for the RNA of heterologous tissue. From cross-reassociation experiments between the DNAs 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. Preliminary data also indicate that repetitive and single copy sequences are interspersed in the DNA of the template-active fraction. These findings are relevant to suggested models of gene regulation (Britten and Davidson, 1971).

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

Figure 1. Fractionation scheme.

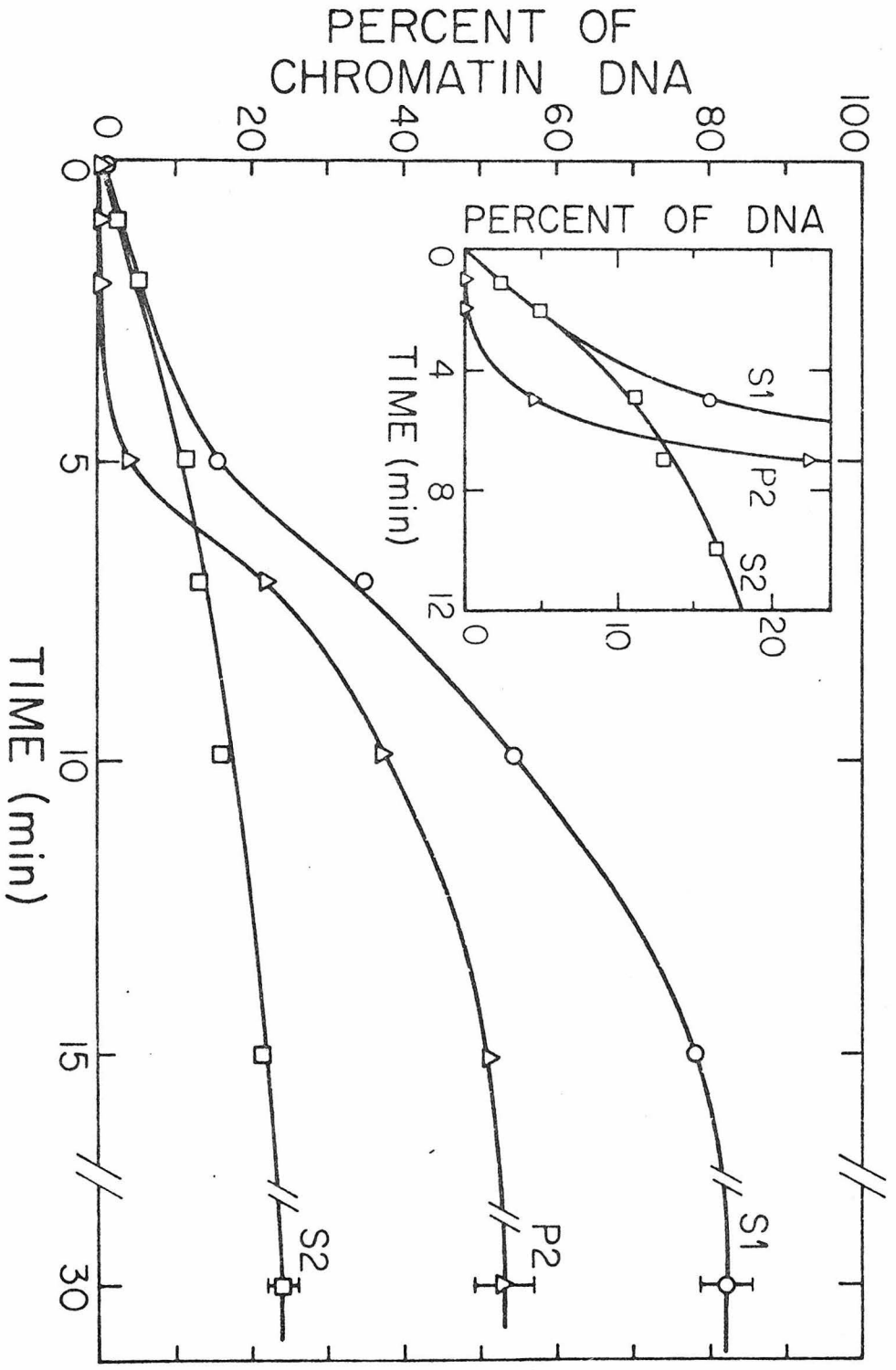

Chromatin (370 μg/ml DNA,
25 mM Na Acetate, pH 6.6)
DNase II
(100 units/ml)
Centrifuge (27,000g, 15 min)
Pellet
P1
Supernatant
S1
2 mM MgCl2
Centrifuge
(27,000g, 15 min)
Pellet
P2
Supernatant
S2

than 1% of the pH 4.8 activity is observed. Undigested chromatin is removed from solution by 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^{++}-insoluble material (P2) is found in fraction S1. On very long times of digestion (ca. 30-90 min), 80% of the chromatin DNA 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; Gottesfeld et al., 1974b; Gottesfeld et al., 1975, in preparation).

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


PERCENT OF
CHROMATIN DNA
0
20
40
60
80
100
0
5
10
15
30
TIME (min)
S1
P2
S2
PERCENT OF DNA
0
4
8
12
TIME (min)
0
10
20
S1
P2
S2

The size of the DNA in fraction S2 was determined after various times of incubation with DNAase II (Figure 3). After 30 sec 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. 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, 60% of the S2 DNA becomes acid soluble while the remaining 40% is recovered as 120 nucleotide fragments (Gottesfeld et al., 1975, in preparation).

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 (1974a). We have obtained similar results with rat DNA prepared from liver chromatin (Figure 4a). 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 fold-back sequences (Wilkes and Bonner, in preparation), moderately repetitive

Figure 3. Length of S2 DNA as a function of time of chromatin digestion with DNAase II. Chromatin from rat liver was prepared and fractionated as described. DNA was isolated from fraction S2. Double-strand lenghts were determined by electron microscopy.

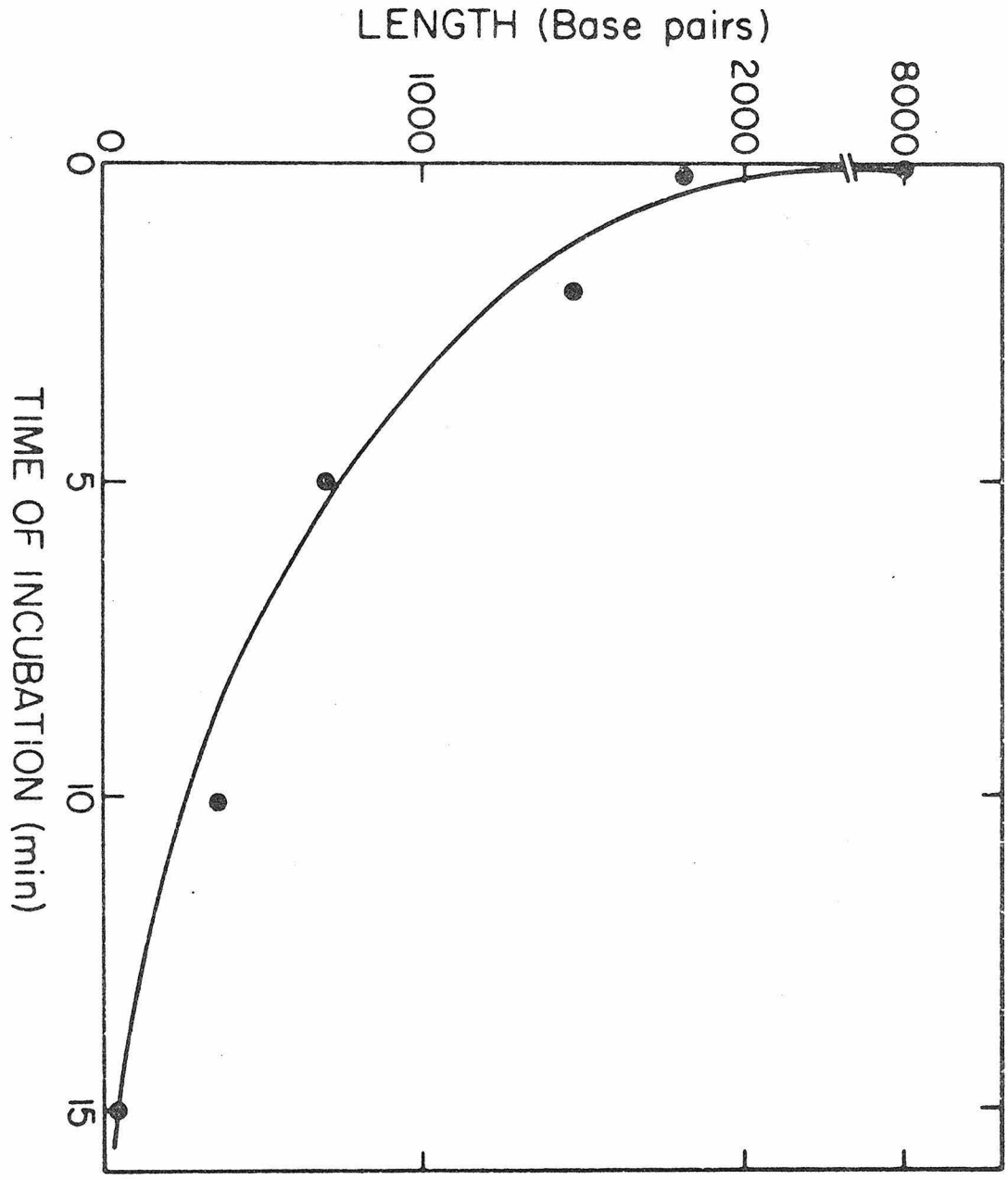
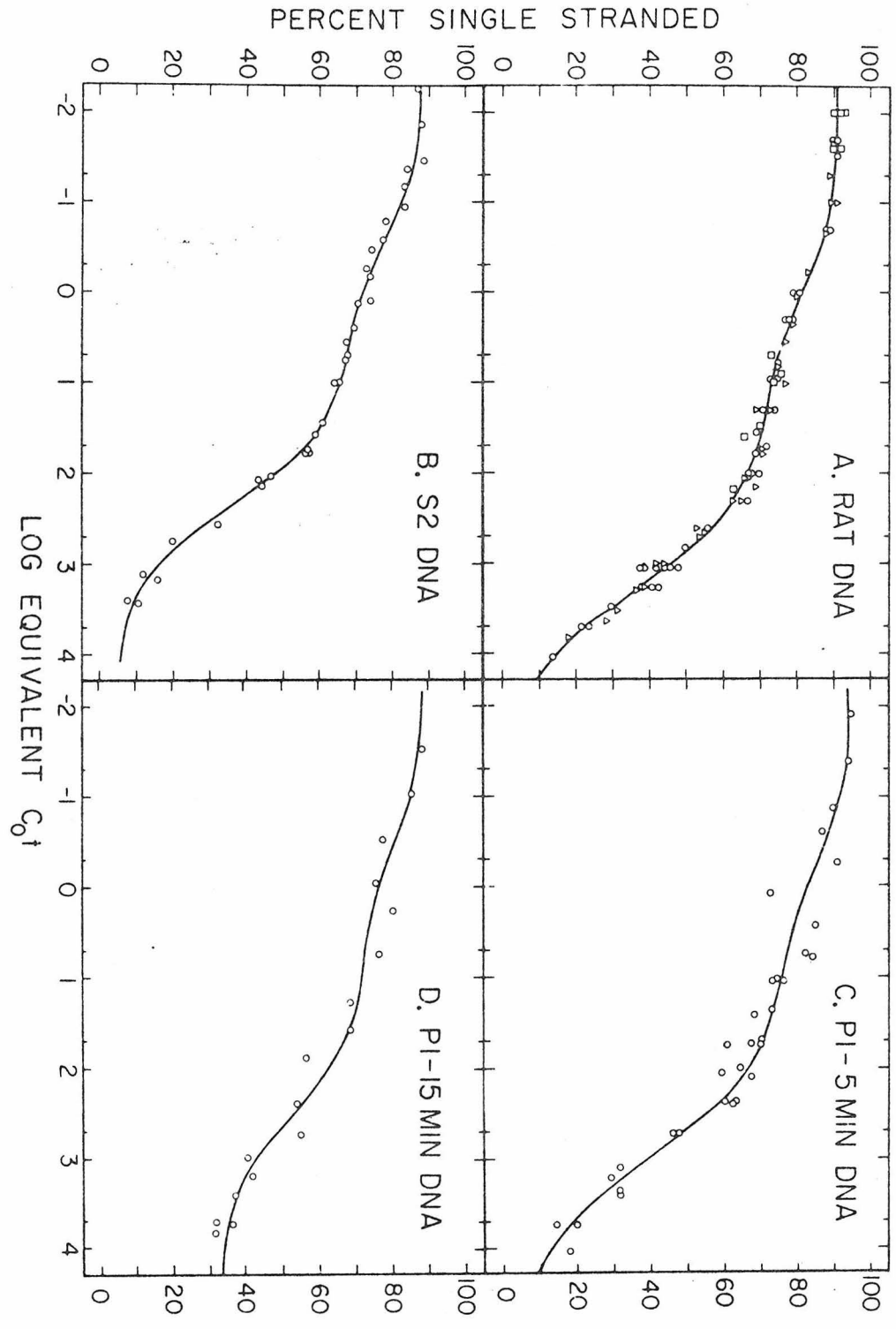

LENGTH (Base pairs)
0
1000
2000
8000
TIME OF INCUBATION (min)
0
5
10
15

Figure 4. 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 $C_o t$ 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 (1974a) 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 versus 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.


PERCENT SINGLE STRANDED
LOG EQUIVALENT C_0t
A. RAT DNA
B. S2 DNA
C. PI-5 MIN DNA
D. PI-15 MIN DNA

sequences which reassociate between $C_o t$ 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, Graham, and Neufeld (1974). The parameters describing the moderately repetitive and single copy components of rat DNA are given in Table 1.

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 (r.m.s.) of a computer analysis with a single middle repetitive component is 3.12% while the r.m.s. 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 ^3H-labeled rat ascites DNA and measured the kinetics of reassociation of this DNA in the presence of an excess of unlabeled rat DNA (Figure 5). The tracer was isolated by standard techniques of reassociation and hydroxyapatite chromatography (Britten and Kohne, 1968). Highly repetitive DNA was removed after incubation to $C_o t$ 0.1; single-stranded DNA was then allowed to reassociate to $C_o t$ 50. The resulting duplex-containing material was used as the middle repetitive tracer. Reassociation takes place over 3-4 log units of $C_o t$ (Figure 5), indicating heterogeneity in the population of middle repetitive sequences. A two-component least-squares analysis of the data has been performed (Figure 5) and the $C_o t$ 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

Table 1. 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	$C_o t$ 1/2 Observed[a]	Estimated $C_o t$ ½ 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	1.0×10^5	3200
		Slow	0.094	32	3.01	3.3×10^6	60
		Nonrepetitive	0.627	2008	1259	1.4×10^9	1.0
				R.M.S. = 3.17%[g]			
Fraction S2 DNA (5 min nuclease exposure)	14.3±3.9[e]	Very Fast	0.109	-	-	-	-
		Intermediate					
		Fast	0.169	0.15	0.025	2.7×10^4	1720
		Slow	0.095	2.76	0.15	1.7×10^5	90
		Nonrepetitive	0.628	235	148	1.6×10^8	1.2
				R.M.S. = 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	8.6×10^4	3160
		Slow	0.175	33	5.8	3.3×10^6	50
		Nonrepetitive	0.638	1706	1037	1.2×10^9	1.0
				R.M.S. = 4.34%[g]			
Fraction P1 DNA (15 min nuclease exposure)	73.9±2.9[f]	Very Fast	0.060	-	-	-	-
		Intermediate					
		Fast	0.104	0.11	0.018	2.0×10^4	5400
		Slow	0.126	14.8	1.86	2.1×10^6	40
		Nonrepetitive	0.340	593	202	2.2×10^8	1
				R.M.S. = 3.95%[g]			

(Footnotes continued)

Table 1 (continued)

[a]Calculated from Figures 4a-4d.

[b]$C_o t$ 1/2 for pure component = $C_o t$ 1/2 observed x fraction of DNA in component.

[c]The complexity of E. coli is 4.2×10^6 base pairs (Cairns, 1963). The $C_o t$ 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:

$$CC = G(fC)(fD)$$

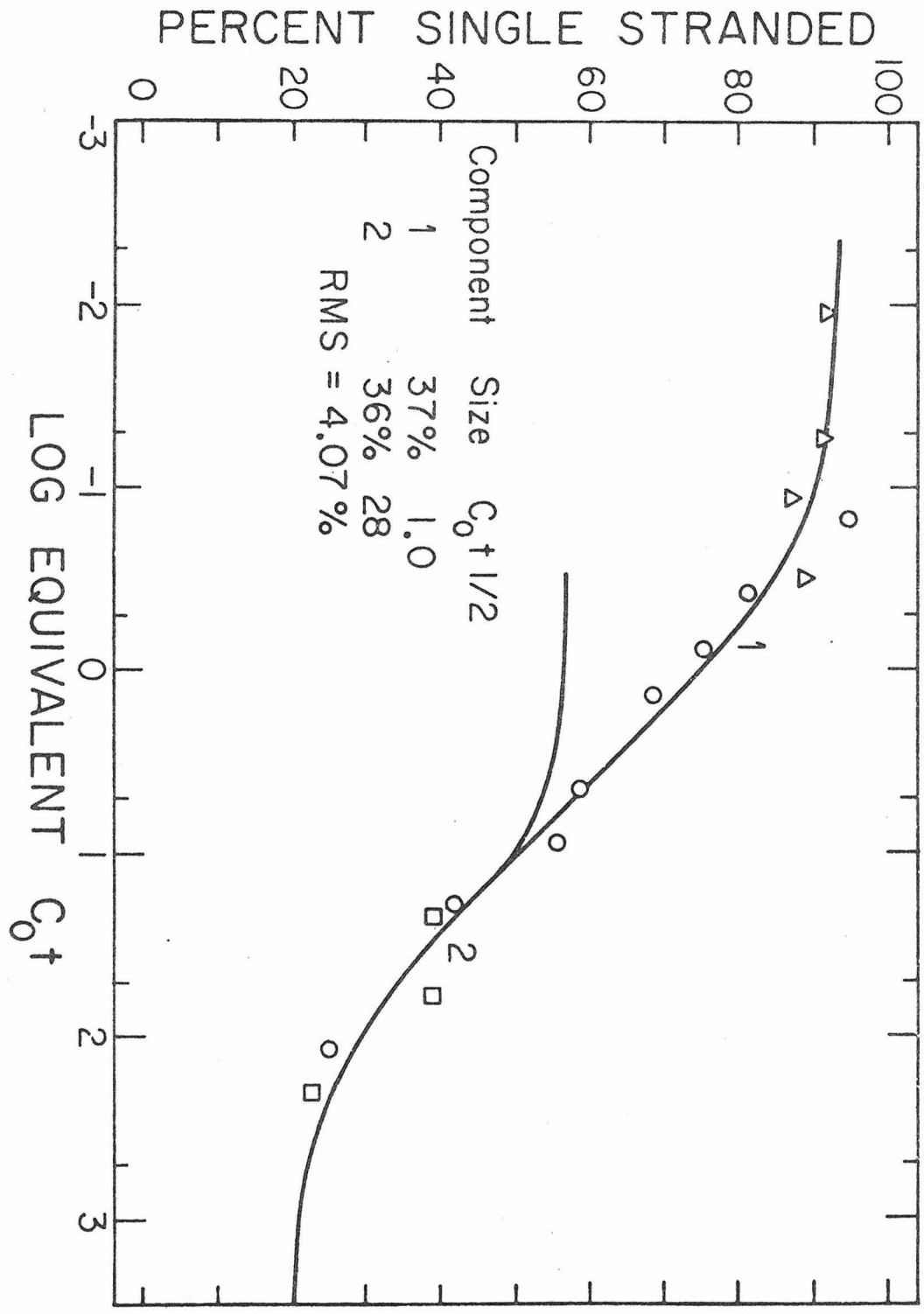
Where CC is the chemical complexity, G is the genome size of the rat (2.7×10^9 base pairs; Sober, 1968), fC is the fraction of total chromatin DNA in a given chromatin sample, fD is the fraction of DNA in the kinetic components. For example, the chemical complexity of the slow component of fraction S2 DNA is (2.7×10^9) (0.113) (0.628) base pairs.

[e]Data of Gottesfeld et al. (1974a)

[f]Data of Gottesfeld et al. (1974b).

[g]Root mean square (r.m.s.) deviation of computer analysis (Britten et al., 1974).

Figure 5. 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 (Experimental) and mixed with a 200-fold excess of 350 nucleotide-long rat liver DNA in 0.12 M PB (0) or 0.48 M PB (□). The abscissa refers to $C_o t$ values of driver rat DNA. The low $C_o t$ points (Δ) were obtained in the absence of driver DNA; tracer $C_o t$ 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. The $C_o t$ 1/2 values of the two components are 1.0 and 27.5. The r.m.s. of the fit is 3.8%.


PERCENT SINGLE STRANDED
0
20
40
60
80
100
-3
-2
-1
0
1
2
3
LOG EQUIVALENT C_0t
Component Size C_0t 1/2
1 37% 1.0
2 36% 28
RMS = 4.07%

1). 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, 60-70 and 2000-3000 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 stranded 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 4b 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 4b has been performed (Britten et al., 1974) and the parameters describing the kinetic components are presented in Table 1. 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 (fold backs). The intermediately and slowly renaturing components of fraction S2 DNA reanneal

at lower $C_o t$ 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 4c and Table 1). 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 hr 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 350 base pairs and reassociation kinetics were determined (Figure 4d and Table 1). About 30% of the DNA failed to reassociate, presumably due to degradation caused by the extensive DNAase treatment. Under standard reassociation conditions (0.18 M Na^+, 62°), 33% of whole rat DNA fragments (350 base pairs in length) contain repeated sequences. On the other hand, 50% of the hybridizable P1 DNA contains repeated

sequences. Thus, P1 DNA appears to be enriched in repeated sequences.* The middle repetitive and nonrepetitive components of P1 DNA reassociate 2 to 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.

Cross-Reassociation Experiments

To test the degree of homology in DNA sequence between the chromatin fractions, we have performed cross-reassociation experiments. Figure 6 depicts the reassociation of labeled P1 DNA in the presence of an excess of either unfractionated chromatin DNA or fraction S2 DNA. When driven by whole chromatin DNA, the labeled P1 DNA tracer reassociates with kinetics essentially identical to the kinetics of the driver. On the other hand, when fraction S2 DNA is used as the driver (upper curve, Figure 6) reassociation is limited to 28% at a driver $C_o t$ of 2000. By this $C_o t$, 90% of S2 DNA has reassociated (Figure 4b). As much as one-third of the observed P1 tracer reassociation could be due to self-reaction since an S2 driver $C_o t$ of 2000 corresponds

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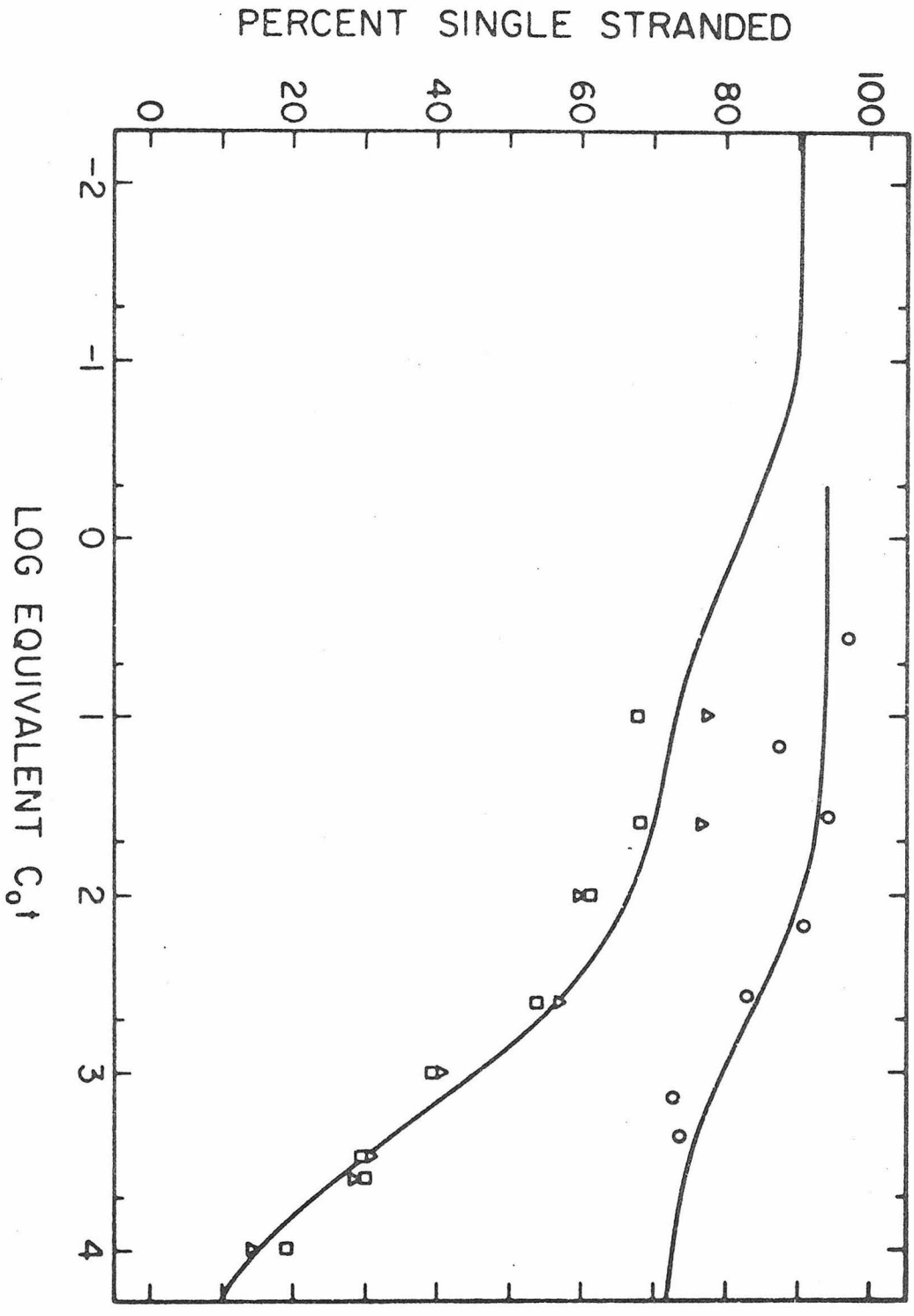
*This conclusion rests on the assumption that the nonhybridizable P1 DNA is a random population of sequences and not specifically single copy sequences. From our knowledge of chromatin structure, it is hard to envisage a mechanism for the specific degradation of single copy DNA.

Figure 6. Cross-reassociation of S2 and P1 DNAs. Chromatin was incubated with DNAase II for 5 min, fractionated and DNA prepared from the fractions as described. Fraction P1 DNA was labeled in vitro with ^{125}I (Commerford, 1971; Holmes and Bonner, 1974a). Fast reassociating material was stripped from the labeled DNA (Experimental). In the lower curve ^{125}I-P1 DNA (□) was mixed with total rat liver chromatin DNA (Δ) at a driver to tracer ratio of 12,800:1. In the upper curve ^{125}I-P1 DNA (0) was mixed with fraction S2 DNA (5 min nuclease treatment) at a driver to tracer ratio of 6250:1. The abscissa refers to $C_o t$ 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 4a). 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 $C_o t$ 1/2 value observed is 600; the reaction goes to 28% with 23% in this single component.


PERCENT SINGLE STRANDED
0
20
40
60
80
100
LOG EQUIVALENT C_0t
-2
-1
0
1
2
3
4

to a tracer $C_o t$ of 0.32 ($C_o t$ 2000 divided by the driver to tracer mass ratio). A $C_o t$ 1/2 of 600 is observed for a single transition in the P1 DNA tracer reassociation. This should be compared to a driver $C_o t$ 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 $C_o t$ 2000 of the S2 driver DNA. If further reassociation was observed at higher $C_o t$ values, this would indicate that P1 sequences are found in low abundance in S2 DNA. With this 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 ($C_o t = 10^4$) or fraction S2 DNA ($C_o t = 5 \times 10^3$) was used as a driver. When fraction P1 DNA was the driver, 53% reassociation of the S2 tracer was observed at a similar driver $C_o t$. The $C_o t$ 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 ($\geq$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, 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. On the other hand, when liver S2 DNA was the driver, the reassociation kinetics of Figure 7 were obtained. Brain S2 DNA reassociated to only 31% at a driver $C_o t$ of 2000. At this $C_o t$, the liver S2 driver DNA reassociated to 90% (lower curve, Figure 7). A computer analysis of the data suggests that brain S2 DNA might reassociate to 40% at higher $C_o t$ values ($>10^4$); however, we have no data to support this. Furthermore, control experiments show that 10% out of the observed 30% reassociation at $C_o t$ 2000 could be due to tracer self-reaction. We take these data to mean that chromatin fractionation is decidedly tissue-specific; we estimate that no more than 25 to 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 4b) 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 8). The labeled

Figure 7. Cross-reassociation of brain and liver fraction S2 DNAs. Chromatin was prepared from brain and liver, 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 (74°C). The abscissa refers to $C_o t$ 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 4b); 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 $C_o t$ 1/2 of 50 and another (23.9%) with a $C_o t$ 1/2 of 1500. The extrapolated final reaction is 41%.

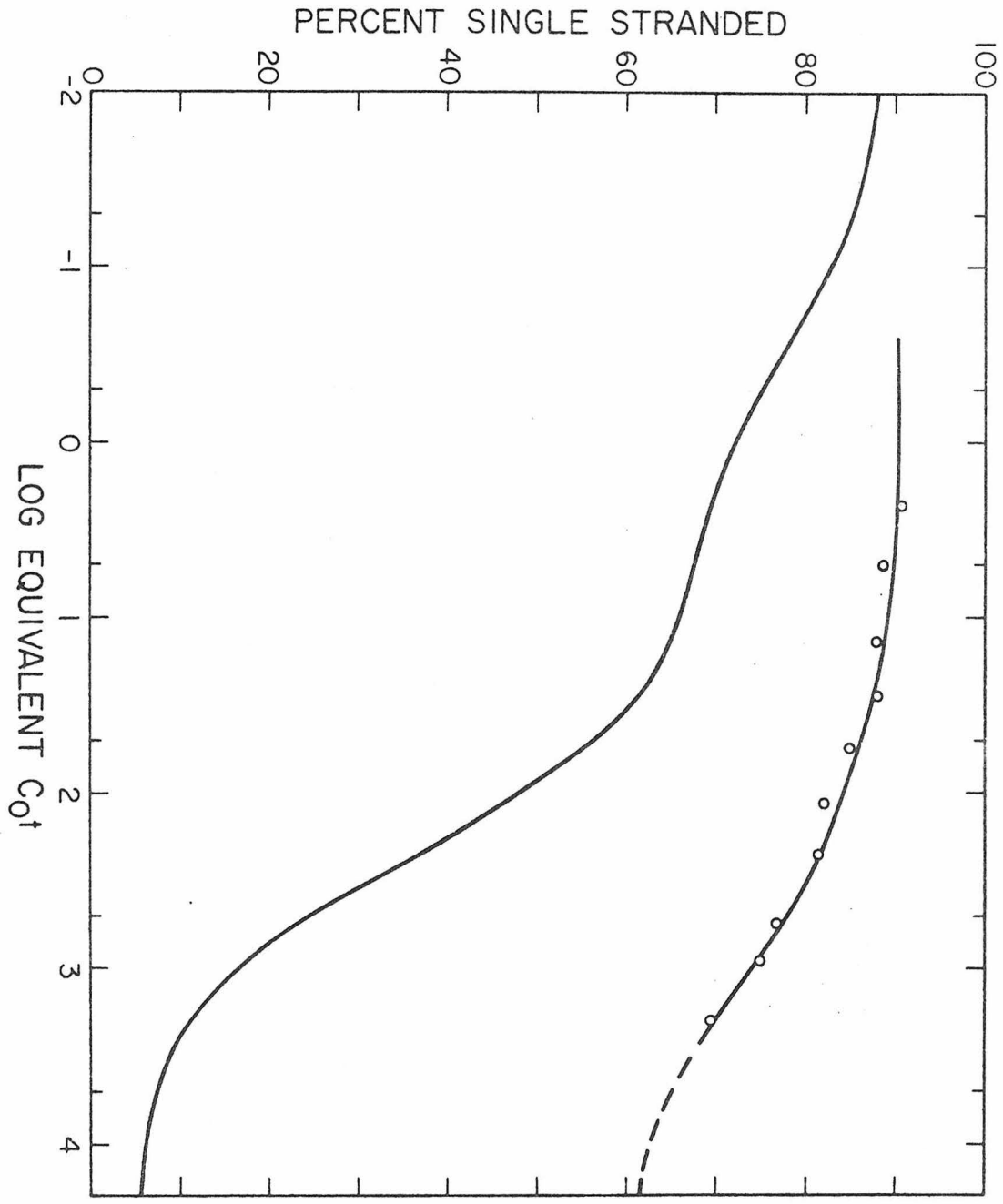
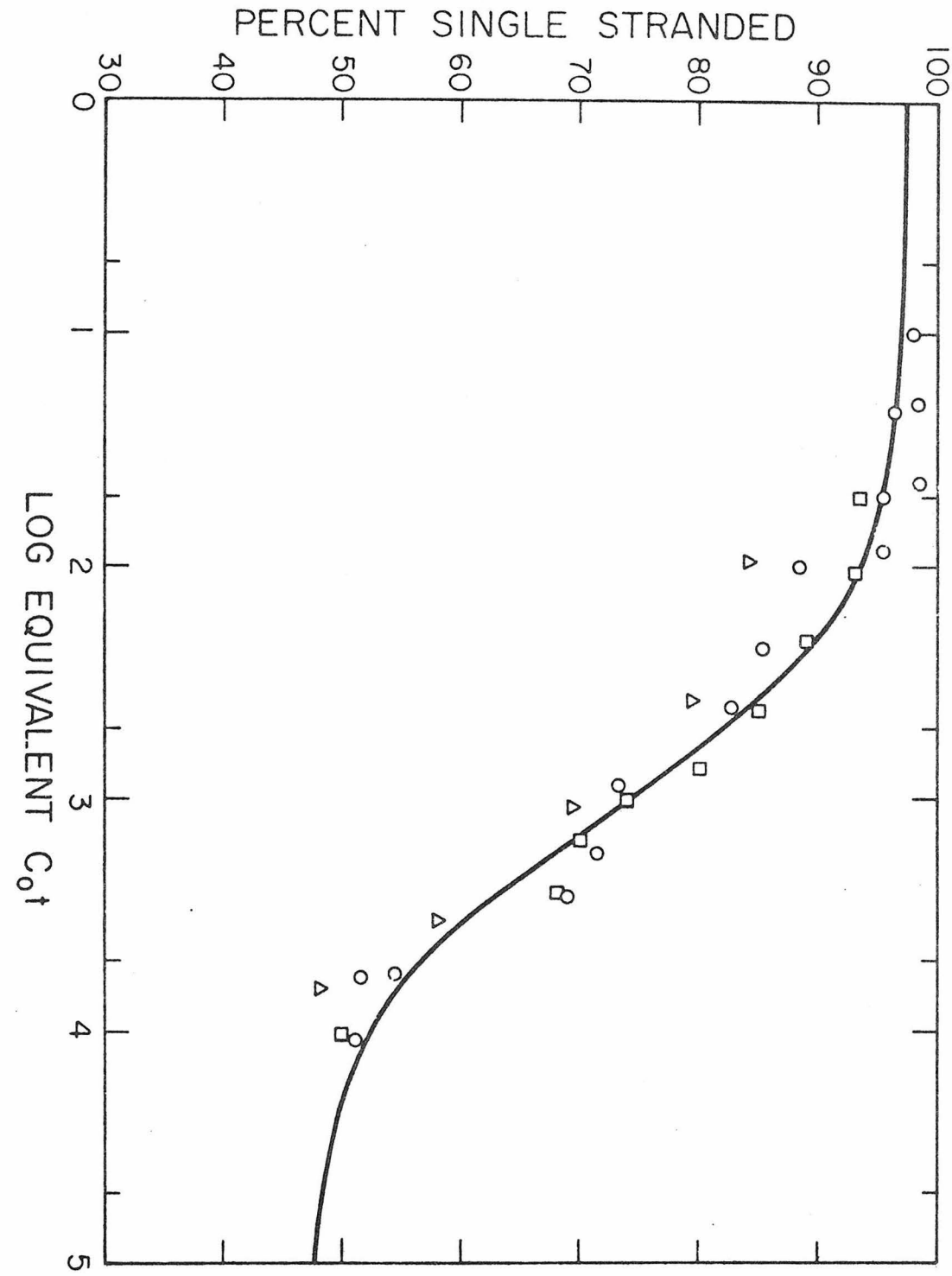

PERCENT SINGLE STRANDED
0
20
40
60
80
100
LOG EQUIVALENT C_0t
-2
-1
0
1
2
3
4

Figure 8. Reassociation profiles of isolated nonrepetitive ^{125}I-labeled DNA from fraction S2 in the presence of an excess of unfractionated DNA. Nonrepetitive DNA from fraction S2 was prepared and labeled as described (Experimental) and mixed with a 2-3 x 10^2-fold excess of 350 nucleotide-long unlabeled rat DNA (O). Labeled DNA was also isolated from DNA-RNA hybrids either with (□) or without (Δ) ribonuclease treatment (see Experimental). The labeled DNAs were mixed with sheared, unlabeled whole rat DNA at a driver to tracer ratio of either 970:1 (□) or 31,000:1 (Δ). The abscissa refers to $C_o t$ values of the driver DNA. The line through the data is a least squares second order fit of the experimental data for the initial tracer DNA (O); the $C_o t$ 1/2 is 1.3 x 10^3 and the root mean square of the fit is 3.1%.


PERCENT SINGLE STRANDED
30
40
50
60
70
80
90
100
0
1
2
3
4
5
LOG EQUIVALENT C_0t

DNA reassociated with only the nonrepetitive sequences of the driver DNA; the $C_o t$ 1/2 observed for the tracer DNA was 1.3×10^3 while the $C_o t$ 1/2 observed for the slow component of driver rat DNA was 2.0×10^3 (Figure 4a). The $C_o t$ 1/2 value of labeled S2 DNA could be in error since terminal data was not obtained (Figure 8); further reassociation would increase the value of $C_o t$ 1/2 for the reaction. However, this would not alter the conclusion that the S2 slow component is derived from single copy sequences of whole rat DNA. The labeled DNA reassociated to only 50% completion, presumably due to breakdown during the several cycles of denaturation and incubation necessary to purify this material (see Experimental). From the reassociation profile of fraction S2 DNA (Figure 4b), it can be calculated that if fraction S2 represents a specific subset of the rat genome (11.3%), then the slow component reassociates at the rate predicted for nonrepeated sequences (Table 1). We may therefore conclude that since this 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, this fraction 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 4b, Table 1) shows 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 9). The middle repetitive DNAs were isolated by standard techniques of reassociation and hydroxyapatite chromatography; $C_o t$ values were chosen to minimize contamination with highly repetitive and nonrepetitive sequences (see Experimental). The DNAs were labeled in vitro by treatment 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 9).

The reassociation of S2 and P1 middle repetitive DNA takes place over four to five log units of $C_o t$ (Figures 9a, 9c). These data confirm the result of Figure 5; 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 $C_o t$ 1/2 values are given in Figure 9. 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 9a and 9c, we calculate that the middle repetitive complexity of fraction S2 is 5-7% that of fraction P1. The kinetic complexities of the components of P1 and S2 middle repetitive DNAs are listed in Table 2. The complexity of the S2 fast reassociating component borders on the complexity of simple sequence DNA. This complexity is 6-8% that of the fast component of P1 middle repetitive

Figure 9. Reassociation curves of ^{3}H-labeled middle repetitive DNAs from fractions P1 and S2 in the presence of excess unlabeled P1 and S2 middle repetitive DNAs. A. Middle repetitive DNA of fraction S2 was labeled by nick translation (Experimental) and mixed with unlabeled S2 middle repetitive DNA at driver to tracer ratios of 1200:1 (0) and 5:1 (□). B. S2 tracer in the presence of unlabeled P1 middle repetitive DNA. The driver to tracer ratios were 2600:1 (0) 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 (0). D. P1 tracer in the presence of S2 middle repetitive DNA. The driver to tracer ratios were 4500:1 (0) and 900:1 (□). All reactions were carried out in 0.12 M PB in sealed capillary tubes. $C_o t$ 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 analysis (Britten et al., 1973). The component sizes and $C_o t$ 1/2 values are given for each curve.

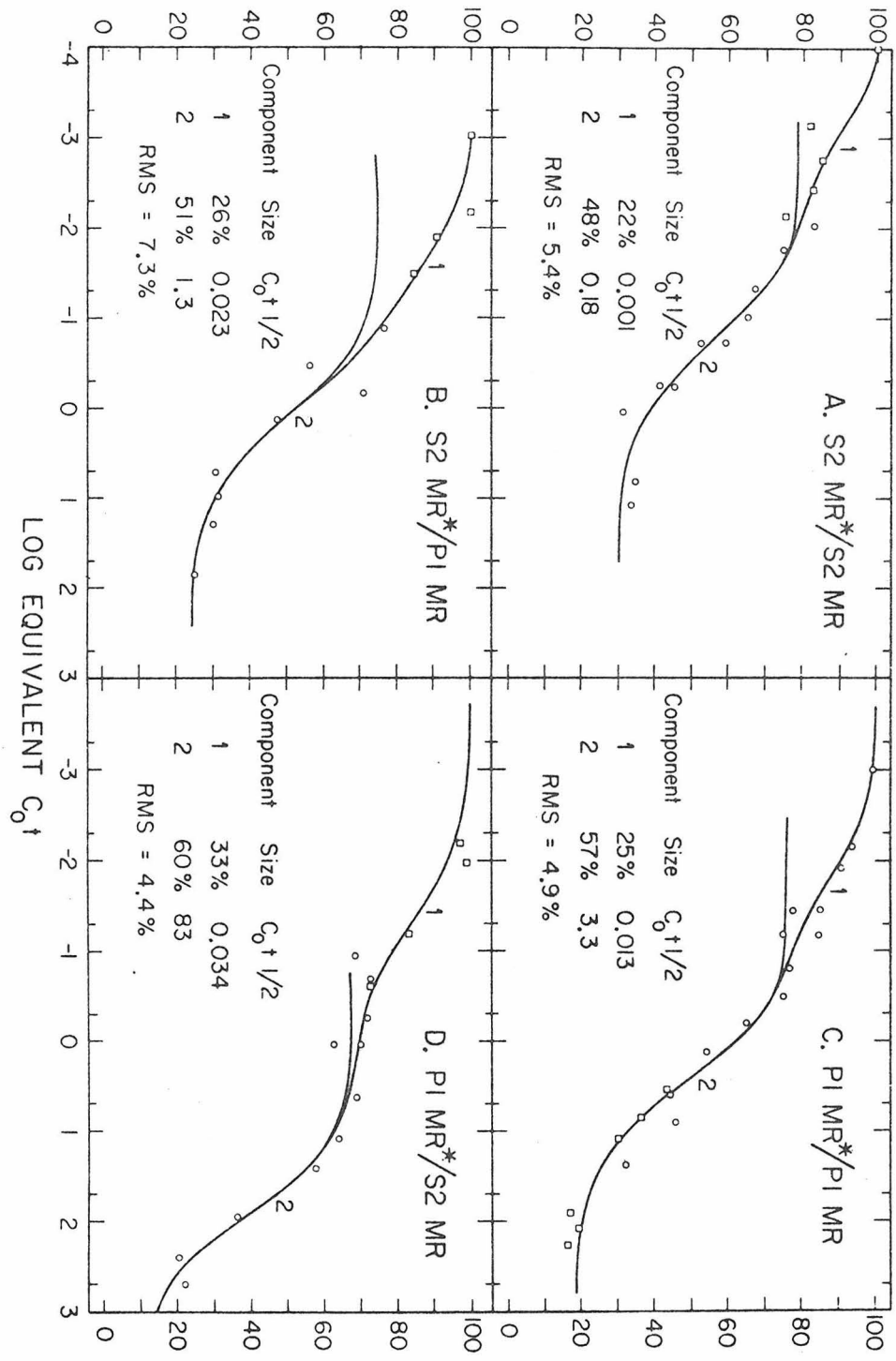

PERCENT SINGLE STRANDED
A. S2 MR*/S2 MR
Component Size C_0t 1/2
1 22% 0.001
2 48% 0.18
RMS = 5.4%
B. S2 MR*/PI MR
Component Size C_0t 1/2
1 26% 0.023
2 51% 1.3
RMS = 7.3%
C. PI MR*/PI MR
Component Size C_0t 1/2
1 25% 0.013
2 57% 3.3
RMS = 4.9%
D. PI MR*/S2 MR
Component Size C_0t 1/2
1 33% 0.034
2 60% 83
RMS = 4.4%
LOG EQUIVALENT C_0t

Table 2. Complexity of Middle Repetitive DNA

DNA of Chromatin Fraction	Complexity of Middle Repetitive Components[a] (base pairs)	
	Fast Component (1)	Slow Component (2)
S2	270	107,000
P1	4000	2,000,000

[a]Kinetic complexity calculated relative to E. coli (see Table 1, footnotes).

DNA. The complexity of the S2 slow repetitive component is 5.2% 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 complexity calculations of Table 2 are more likely to be accurate than those of Table 1 since the present calculations are based on the reassociation curves of isolated components. The calculations of Table 1 are based on computer analyses of small regions (<25%) of the reassociation curves (Figure 4). Furthermore, scatter of the experimental points (especially Figure 4c) makes calculations based on these data tenuous.

The reassociation experiments of Figures 9b and 9d 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 9b were obtained. This reassociation curve is essentially identical to the reassociation curve of P1 middle repetitive DNA (Figure 9c). 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. The reassociation kinetics of Figures 9a and 9b allow us to calculate that S2 repetitive sequences make up between 4.4 and 13.8%

of the complexity of P1 middle repetitive DNA. This calculation was made by comparing the $C_o t$ 1/2 values and sizes of the kinetic components. If we compare the overall $C_o t$ values of the curves of Figures 9a and 9b, we calculate that S2 sequences comprise 11% of P1 middle repetitive complexity.

The data of Figure 9d 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 9c and 9d), fraction S2 must contain all P1 sequences. We note, however, that during the last phase of reassociation (at $C_o t$ 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%, $C_o t$ 1/2 = 0.034) reassociates over the same range of $C_o t$ values as the major portion of S2 driver reassociation ($C_o t$ 0.005 to $C_o t$ 1.0). The second component (60%, $C_o t$ 1/2 = 83) reassociates nearly 500 times slower than the slow component of the S2 driver ($C_o t$ 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 ($\frac{0.18}{83}$ = 0.2%). This could be an underestimate since P1 sequences in S2 DNA might have been partially removed by the $C_o t$ 2 fractionation step used in the preparation of S2 middle repetitive DNA (see Experimental). The data of Figure 9d suggest that about one-third of the <u>mass</u> of P1 repetitive DNA has complements

in S2 DNA at significant concentrations. The experiment depicted in Figure 9b showed that about 11% of the complexity of fraction P1 repetitive DNA is due to the presence of S2 repetitive sequences. Table 3 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 10. 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 5 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. This experiment is described in detail above. When the middle repetitive tracer was used with unlabeled S2 DNA as a driver, the results of Figure 10 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 four logs of driver $C_o t$. A two component least squares fit of the data is shown in Figure 10.

Table 3. Composition of Middle Repetitive DNA of Chromatin Fractions P1 and S2[a]

Chromatin Fraction[b]	$C_o t$ 1/2 of Repetitive Component					
	Fast Repetitive			Slow Repetitive		
	0.001	0.013	0.034	0.18	3.3	83
	Percent of Repetitive DNA in Component (by mass)					
S2	22% (9a)[c]	26% (9b)	possible 10-20% (9a)	48% (9a)	51% (9b)	0.2-5%[d] (9a)
P1	<2-4% (9c,d)	25% (9c)	33% (9d)	possible 5-10% (9c,d)	57% (9c)	60% (9d)

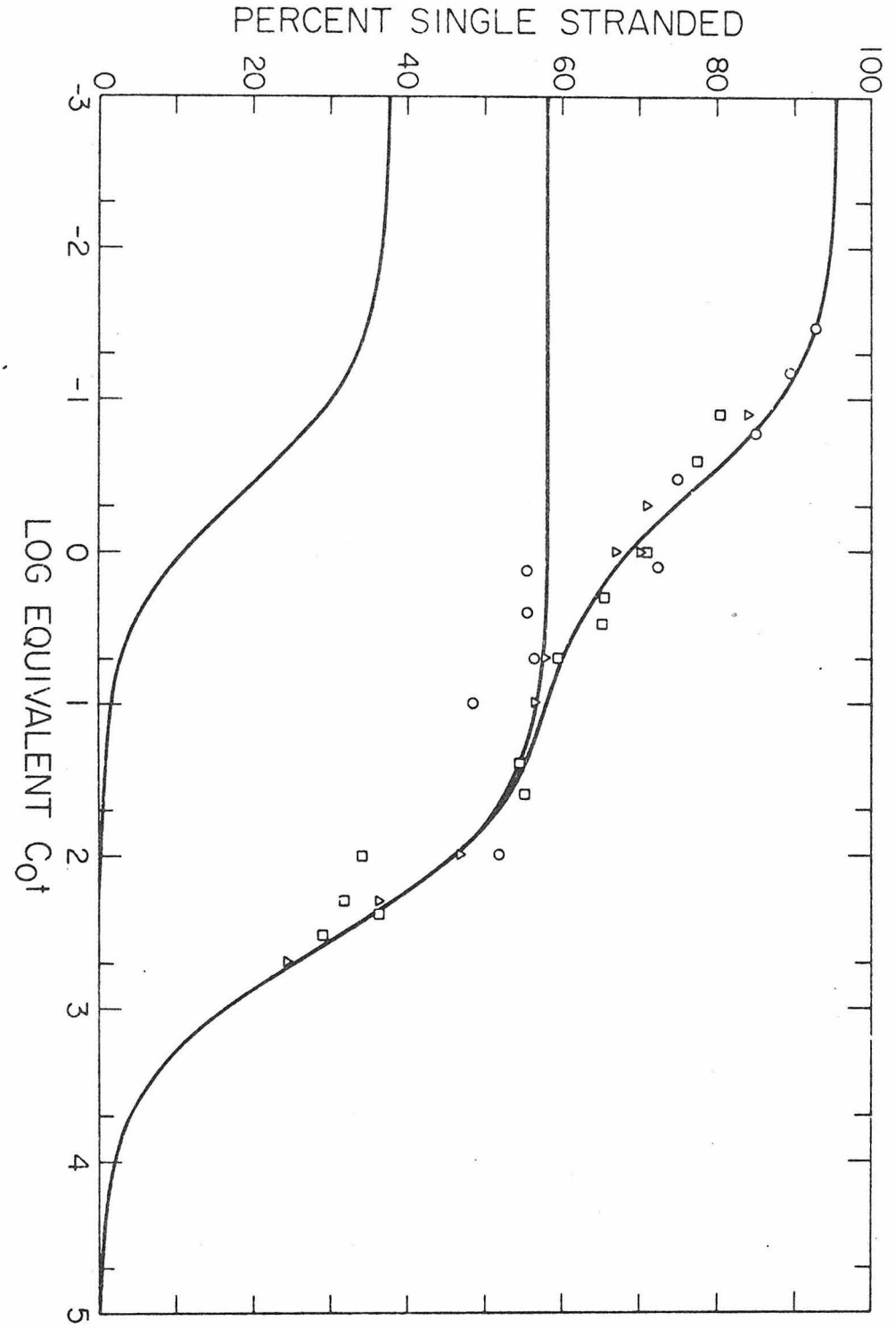
[a]From data of Figures 9a-d.

[b]Chromatin fractions were obtained after 5 min of nuclease treatment.

[c]Indicates that this estimate was obtained from Figure 9a, etc.

[d]This is an underestimate since some of this component, if present in S2 DNA, would have been lost from isolated S2 middle repetitive DNA by $C_o t$ 2 fractionation (see text).

Figure 10. 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 (0,Δ). The ratio of unlabeled S2 DNA to tracer repetitive DNA was 2000:1 (0), 1400:1 (Δ), 3000:1 (□). The abscissa refers to S2 DNA driver $C_o t$ 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 $C_o t$ 1/2 of 0.33, and component 2 (58%) has an observed $C_o t$ 1/2 of 380.


PERCENT SINGLE STRANDED
0
20
40
60
80
100
LOG EQUIVALENT C_0t
-3
-2
-1
0
1
2
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4
5

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 $C_o t$, the families of middle repetitive sequences are present at various levels of abundance in S2 DNA. From the fit of the data (Figure 10), 37% of the hybridizable tracer reassociates over the same range of $C_o t$ values as the intermediate components of fraction S2 DNA ($C_o t$ 0.02 to $C_o t$ 10). The second component (58%) exhibited a $C_o t$ 1/2 of 380. This value is nearly the $C_o t$ 1/2 value for nonrepetitive DNA in the S2 driver DNA ($C_o t$ 1/2 = 225, Table 2). 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 per haploid genome), while the major fraction of repetitive sequences (58%) is represented only once or a few times in fraction S2 DNA.

DNA-RNA Hybridization

We have shown previously (Gottesfeld et al., 1974a) that fraction S2 is enriched by a factor of 4 to 5 over fraction P1 in nonrepetitive sequences which code for cellular RNA. This was shown by hybridizing ^{125}I-labeled nonrepetitive DNA from liver chromatin fractions to an excess of total cellular RNA from liver. In RNA-driven hybridization experiments of this sort a simple saturation curve is obtained (Figure 4 of Gottesfeld et al., 1974a). The amounts of DNA found in DNA-RNA hybrids in our previous experiments are given in Table 4. Since the different tracer preparations used reassociated to

Table 4. DNA-RNA Hybridization Experiments with Liver Chromatin Fractions

RNA	Liver Chromatin Nonrepetitive DNA[a]	RNA Saturation Observed[b] (% of DNA in DNA-RNA hybrid)	Final Reaction of DNA Tracer[c] (% Double Stranded)	Corrected RNA Saturation[d] (% of DNA in DNA-RNA hybrid)
Liver	S2	14.5[e]	52.2[e]	27.8
Liver	P1	3.5[e]	67.2[e]	5.2
Brain	S2	9.6	69.5	13.7
Brain	P1	8.9	67.2[e]	13.2

[a]Nonrepetitive DNA was isolated from the chromatin fractions S2 and P1 (5 min nuclease incubation) and labeled in vitro with ^{125}I (Commerford, 1971; Holmes and Bonner, 1974).

[b]DNA tracers were mixed with whole cell RNA (see Experimental) at a ratio of 2-2.5 x 10^4:1 (RNA:DNA) in 0.03 M PB, 0.675 M NaCl, denatured for 5 min at 110°C, and incubated at 74°C for 2-4 days. R_ot values of 5.6-11.2 x 10^4 were obtained. Saturation is obtained after 1-2 days ($R_ot > 2.8 \times 10^4$). The amount of labeled tracer in hybrid form was determined as described in Experimental section.

[c]DNA tracers were allowed to reassociate in the presence of an excess of unlabeled 350 nucleotide-long unfractionated DNA, and the amount of DNA in hybrid assayed at high C_ot (1 x 10^4).

[d]Corrected saturation value is obtained by dividing the observed saturation value by the fraction of the tracer DNA which was able to form hybrids.

[e]Data of Gottesfeld et al. (1974).

various extents when driven by whole rat DNA, we have corrected the RNA saturation values for the extent of hybridizable DNA in each preparation. Thus, fraction S2 is enriched in template-active sequences over fraction P1 by a factor of 5.4.

Since only a minor fraction of the tracer DNA ever forms hybrids with RNA, it is important to characterize this DNA. We have isolated the DNA from DNA-RNA hybrids by the method of Galau, Britten, and Davidson (1974). In this method, duplex-containing DNA is isolated first by hydroxyapatite chromatography, and then incubated with RNAase. Any tracer DNA which formed DNA-DNA duplexes would still be double-stranded after this treatment; however, DNA from DNA-RNA hybrids becomes single stranded after RNAase treatment and thus separable from DNA-DNA duplexes by hydroxyapatite chromatography. The extent of DNA-DNA duplex formation during the hybridization experiment was found to be 2-3%. This is consistent with the value reported earlier (Gottesfeld et al., 1974a) obtained from a self-reaction of tracer in the presence of base-hydrolyzed RNA. The DNA isolated from DNA-RNA hybrids was mixed with an excess of 350 nucleotide-long rat DNA and reassociation kinetics of the tracer determined (squares, Figure 8). The reassociation curve of the tracer isolated from DNA-RNA hybrids is essentially identical to the reassociation profile of the tracer preparation prior to DNA-RNA hybridization. This shows that the DNA which formed hybrids with RNA was nonrepetitive DNA and not a minor repetitive contaminant of the tracer preparation. We do not know why the DNA which had been in duplex with RNA failed

to reassociate to completion when driven with whole rat DNA, although this DNA is probably of small size after the many cycles of denaturation and incubation at high temperature to which it has been subjected. We have also used the total duplex containing DNA after DNA-RNA hybridization in a similar experiment (triangles, Figure 8). DNA-DNA duplexes comprise 17% of this material (3%/3% + 14.5%). When driven by whole rat DNA, the total duplex-containing DNA exhibited a significant degree of reassociation at $C_o t$ values prior to the reassociation of nonrepetitive sequences ($C_o t < 10^2$). These rapidly reacting sequences comprise 21% of the hybridizable material in this preparation (14%/64% final reaction from a computer fit of the data). Thus the fast reacting material could correspond to the DNA which formed DNA-DNA duplexes during the RNA-driven reaction.

Melting profiles of reassociated nonrepetitive tracer DNA from fraction S2 have been determined (data not shown). Tracer was allowed to anneal to either whole rat DNA or rat liver RNA to high $C_o t$, and unreassociated material was separated from duplex-containing material by hydroxyapatite chromatography. Melting curves were then determined on the material absorbed to hydroxyapatite. The T_m of unlabeled reassociated rat DNA was 84°; the T_m of the reassociated iodinated nonrepetitive tracer was 80-81°. The T_m of the DNA which hybridized to RNA was similar (17% of this material is in the form of DNA-DNA duplexes). We do not know the reason for this decrease in T_m although it might be attributed to decrease of tracer size due to the several cycles of denaturation and incubation necessary to prepare this material,

or to the iodination procedure.

We have also measured the extent of duplex formation of nonrepetitive tracer DNAs in the presence of an excess brain RNA (Table 4). The tracer DNAs were isolated from the liver chromatin fractions S2 and P1. The percentage of tracer in DNA-RNA duplex (corrected for DNA-DNA reassociation) was nearly the same for the two chromatin fractions. This indicates that fraction S2 DNA isolated from liver chromatin is not enriched over fraction P1 in sequences that code for brain RNA. This finding is consistent with the cross-reassociation experiment of Figure 7. The saturation values obtained with brain RNA are somewhat higher than values reported in the literature for mouse brain RNA (Brown and Church, 1972; Grousse, Chilton, and McCarthy, 1972). If these latter values are corrected for the amount of hybridizable DNA present in the tracer preparation (as we have done), then our values are identical to those in the literature.

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, Allfrey, and Mirsky, 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 have reported previously (Gottesfeld et al., 1974a) that the active fraction S2 is enriched in nonhistone chromosomal proteins and depleted in histone protein. Histone I (f1) appears to be totally absent from the active fraction. This latter result is in agreement with the findings of Simpson and Reeck (1973) for active chromatin prepared by ECTHAM-chromatography. 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.

McConaughy and McCarthy (1972) have fractionated pressure-sheared chromatin by thermal elution from hydroxyapatite. These authors

have shown by RNA-excess hybridization that the early melting fractions are enriched in DNA sequences transcribed in vivo. This procedure, however, has a major drawback: thermal chromatography destroys native DNA-protein interactions and thus only the active DNA is recovered by this method. An understanding of gene regulation in eukaryotes will require knowledge about both the protein and DNA constituents of active chromatin. We have shown in this paper and previously (Gottesfeld et al., 1974a) that the nuclease-sensitive, Mg^{++}-soluble fraction of liver chromatin is enriched fivefold in nonrepetitive DNA sequences which are transcribed in vivo. This was demonstrated by RNA-excess hybridization with labeled nonrepetitive DNA as tracer. It was also shown that the DNA which actually formed hybrids with RNA was derived from the nonrepeated portion of the rat genome (Figure 8). We have shown that the active fraction from liver chromatin is not enriched in single copy sequences complimentary to brain RNA. Similarly, cross-reassociation experiments with fraction S2 DNA from both liver and brain chromatin demonstrate that a low proportion of brain active sequences are present in the liver active fraction (Figure 7). 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.

An estimate of the proportion of transcribed sequences in fraction S2 may be obtained from the RNA saturation data (Table 4). If all

the nonrepetitive DNA of fraction S2 were transcribed, and transcription was from only one strand, then 50% of the hybridizable tracer should form duplexes with RNA. We found that 29% of the hybridizable tracer DNA from fraction S2 formed DNA-RNA hybrids. This suggests that fraction S2 is 58% "pure" in transcribed nonrepetitive sequences. If the extent of nonrepetitive transcription in the liver is 3-5% (Grouse et al., 1972; Brown and Church, 1972), then we have purified transcribed sequences by a factor of 6 to 10 ($\frac{29}{5}$ to $\frac{29}{3}$) over unfractionated chromatin.

From cross-reassociation experiments with DNAs from liver chromatin fractions, we estimate that fraction S2 is contaminated with fraction P1 sequences to the extent of about 30-40%. This value is derived from the degree of reassociation of a labeled P1-DNA tracer in the presence of an excess of unlabeled S2 DNA (Figure 6). In the converse experiment, about 70% of a labeled S2-DNA tracer reassociated with an unlabeled P1-DNA driver. We interpreted this latter result to mean that not all sequences of S2 DNA are removed from fraction P1 after short times of incubation with DNAase. Indeed, this can be seen in the data of Figure 2; after 5 min of incubation with the nuclease, only half of the S2 DNA is separated from fraction P1.

From the kinetics of reassociation of fraction S2 DNA (Figure 4b), 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 (Figure 8), we calculate that this component comprises 11% of the total single copy complexity

of the rat. The data of Figure 4b also suggest that fraction S2 contains a subset of repeated sequences. This notion is substantiated by the data of Figure 9: the complexity of S2 middle repetitive sequences is about 5-7% that of P1 middle repetitive sequences (Table 2). The cross-reassociation experiment of Figure 9b shows 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 2).

The experiment of Figure 9d 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 $C_o t$ values at which S2 DNA reassociates ($C_o t$ 0.05 to $C_o t$ 1). The remaining P1 repetitive DNA reassociates at much higher $C_o t$ 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 9b 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 $C_o t$ 2 fractionation step used in preparing S2 repetitive DNA could have reduced the concentration of P1 sequences in S2 DNA. We calculate that 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.

Preliminary data suggest that repetitive and single copy sequences are interspersed to a high degree in fraction S2 DNA (Gottesfeld and Bonner, unpublished). This notion comes from an experiment of the type first performed by Davidson et al. (1973). Labeled 1800-nucleotide long S2 DNA (30 sec enzyme treatment) was allowed to reassociate in the presence of an excess of 350-nucleotide long whole rat DNA; after incubation to $C_o t$ 10 and hydroxyapatite chromatography, 70% of the hybridizable tracer was found in the 0.48 M PB fraction. In comparison, when labeled 400-nucleotide long S2 DNA was incubated with whole DNA to the same $C_o t$, only 30% of the DNA was in the hybrid fraction. This suggests that both repetitive and single copy sequences

are present on the majority of 1800-nucleotide long S2 DNA molecules. This result is not surprising since short period interspersion of repetitive and single copy sequences is a phenomenon common to a wide range of organisms (Davidson et al., 1975a) including rat (Bonner et al., 1973). Furthermore, Davidson et al. (1975b) have found that transcribed nonrepeated sequences in the sea urchin tend to be adjacent to repetitive sequences. 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 and nonrepetitive sequences are probably interspersed with repetitive sequences in fraction S2. This suggests that specific repetitive sequences might lie adjacent to transcribed sequences. At present, we do not know to what extent the repetitive sequences of fraction S2 are transcribed.

It is likely that fraction S2 codes primarily for nuclear transcripts (hnRNA). This class of RNA represents about 10% of the total complexity of the rat genome in ascites cells (Holmes and Bonner, 1974a). Messenger RNA complexities tend to be about one-tenth that of hnRNA (Galau et al., 1974; Davidson and Britten, 1973; Hough et al., 1975; Holmes and Bonner, personal communication). Thus, about one-tenth of fraction S2 DNA probably codes for translated polysomal messenger RNA, while the bulk of the S2 DNA codes for untranslated nuclear RNA. Holmes and Bonner (1974b) and Smith et al. (1974) have shown that many repetitive and nonrepetitive sequence transcripts are on the same hnRNA

molecules; that is, hnRNA is transcribed from interspersed repetitive and single copy sequences. Messenger RNA, however, is primarily the transcript of single copy sequences (Davidson and Britten, 1973). Thus, the sequence organization of S2 DNA reflects the sequence organization of hnRNA.

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; however, we hasten to point out that these results should not be taken, at this time, as a confirmation of the Britten-Davidson model.

Experimental

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.

Rat DNA Preparations

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. 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 p.s.i. DNA was sheared in moderate salt (either 0.12 M PB 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 pipette and transferred to another tube above an equal volume of fresh Tris-saturated phenol. This process was repeated five 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 hr. NaCl was added to 0.5 M and sodium dodecyl sulfate to 1%. Pronase (autodigested for 1 hr at 37°C) was added to 25 μg/ml and the mixture was incubated at 60°C for 1-2 hr. 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, Simon, and Davidson (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 $C_o t$ 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 $C_o t$ of 50, and double stranded DNA was separated 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 x 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 $C_o t$ values were used. Highly repetitive DNA was removed from sheared P1 DNA after incubation to $C_o t$ 0.05. The single strand fraction

from a hydroxyapatite column was then allowed to reassociate to $C_o t$ 20 and the double strand fraction was collected. Highly repetitive DNA was removed from S2 DNA after incubation to $C_o t$ 0.02, and middle repetitive DNA was isolated after incubation to $C_o t$ 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, Pl middle repetitive DNA was incubated to $C_o t$ 55 (without denaturation before incubation); S2 middle repetitive DNA was incubated to $C_o t$ 12. These $C_o t$ values are approximately 250-300 times the $C_o t$ 1/2 values calculated for pure middle repetitive DNAs. After incubation, the DNAs were dialyzed at 4° 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/mmole, Nuclear Dynamics) plus 10 μmoles 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° for 22-27 hr, 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° (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 per 20 µl (when counted in 5 ml of Aquasol). The column was then eluted with 0.12 M 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 to 3.5 x 10^6 cpm/µg DNA were obtained. Labeled middle 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.

Nonrepetitive DNA. Chromatin from rat liver was treated with DNAase for 5 min and fractionated as described above. DNA was prepared from fractions S2 and P1. The P1-DNA was pressure sheared as described above. The DNA was denatured and incubated in 0.48 M PB to an equivalent $C_o t$ of 2.5 x 10^2 (S2-DNA) or 1.5 x 10^3 (P1-DNA), and the single stranded fraction was isolated by hydroxyapatite chromatography. This material was dialyzed, lyophilized, dissolved in 0.48 M PB, and allowed to renature as before. The DNA that remained single stranded after two cycles of purification was allowed to renature to a $C_o t$ of 10^4. The resulting duplex-containing DNA was isolated by hydroxyapatite chromatography, dialyzed and concentrated by lyophilization. The purified nonrepetitive DNA was labeled in vitro by the modified Commerford method. Specific activities of 1-10 x 10^6 cpm/µg were obtained.

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 $C_o t$ 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 $C_o t$ values reported herein have been normalized to $C_o t$ 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 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.

RNA-Driven DNA-RNA Hybridization Experiments

Total cellular RNA from rat liver and brain (Pel-Freeze frozen tissue) was isolated by a modified hot phenol-sodium dodecyl sulfate extraction procedure (Moriyama et al., 1969). The RNA was sheared to a weight-average length of 1000 nucleotides by two passes through the Ribi-Sorvall pressure cell at 30,000 p.s.i. The RNA size was determined under nondenaturing conditions in the analytical ultracentrifuge. The Spirin equation was used to relate $S_{20,w}$ to molecular weight of RNA (Spirin, 1963). Mixtures of sheared RNA and nonrepetitive DNA were heat denatured and incubated in 0.03 M PB, 0.675 M NaCl, 1 mM EDTA, pH 6.5, at 74°C. The ratio of RNA to ^{125}I-labeled DNA was 2-2.5 x 10^4:1, and the RNA concentration was 20 mg/ml. Reactions were carried out in sealed capillary tubes. Hybridization reactions were terminated by diluting the samples 20-fold in 0.03 M PB, 0.1 M NaCl at 60°C followed by application to a hydroxyapatite column equilibrated in the same buffer at 60°C. About 1 ml of packed hydroxyapatite was used per 100 µg of RNA. At 0.03 M PB, 0.1 M NaCl, all nucleic acids are absorbed to the resin. Single stranded DNA and the bulk of the RNA is eluted in 0.12 M PB; DNA-RNA hybrids, DNA-DNA duplexes, and about 20% of the input RNA is eluted in 0.48 M PB. The samples were precipitated and counted as described above.

The extent of DNA-DNA self-reaction was estimated in two ways. Ten mg of liver RNA was hydrolyzed in 300 µl of 1.125 N NaOH for 3 hr at 37°C. At the end of this time 200 µl of 1.69 N HCl, 0.075 M PB was added to give a final NaCl concentration of 0.675 M and PB concentration of 0.03 M. A small amount of ^{125}I-labeled nonrepetitive DNA (in distilled water) was added, and the mixture was lyophilized. The dried material was taken up in 500 µl of distilled water; 50 µl aliquots were sealed in capillary tubes, denatured and incubated at 74°C. The amount of DNA in duplex form was estimated as described above. DNA-DNA duplex formation at zero-time was approximately 2%; this amount did not increase during a 96 hr incubation. The second method of estimation of DNA-DNA duplex formation during RNA-driven hybridization was the method of Galau et al. (1974). DNA-RNA hybridization in RNA excess was carried out as described above. The 0.48 M PB fraction from a hydroxyapatite column was dialyzed against 6 ℓ of 0.05 M PB; RNAase A (preincubated at 80°C for 10 min) was added to 10 µg/ml. The mixture was incubated overnight at 37°C and a second hydroxyapatite column was run. The material which elutes in 0.12 M PB is the DNA which was in DNA-RNA hybrid, and the DNA in the 0.48 M PB fraction is in DNA-DNA duplex. By this method, DNA-DNA reassociation was estimated to be 2-3% at high $R_o t$ values. For the isolation of larger amounts of DNA from DNA-RNA hybrids, 2 µg of ^{125}I-labeled S2 nonrepetitive DNA was mixed with 0.4 mg of liver RNA and hybridization was allowed to proceed for 48 hr ($R_o t$ = 23,600). This corresponds to a tracer $C_o t$ of 118; 5% of the input DNA was in DNA-DNA duplex

and 13% of the input DNA was in DNA-RNA hybrid.

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 ØX174 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 hr 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 5.4S under alkaline conditions.

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V. STRUCTURE OF TRANSCRIPTIONALLY-ACTIVE CHROMATIN

To be submitted for publication in the Proceedings of the National Academy of Sciences.

ABSTRACT Rat-liver chromatin has been fractionated into transcriptionally-active and inactive regions [Gottesfeld *et al.* (1974) *Proc. Nat. Acad. Sci. USA* 71, 2193-2197] and the distribution of nuclease-resistant complexes in these fractions has been investigated. About half of the DNA of both fractions is resistant to attach by the endonuclease DNase II. The nuclease-resistant structures of inactive chromatin are DNA-histone complexes (ν-bodies) which sediment at 11-13S. Two nuclease-resistant species are found in active chromatin. These particles sediment at 15 and 20S, respectively, and contain DNA, RNA, histone and nonhistone chromosomal proteins. Thermal denaturation studies suggest that the DNA of the active fraction is complexed with nonhistone protein.

A regular repeating unit in chromatin was first suggested from the X-ray diffraction studies of Pardon, Richards and Wilkins (1): A series of reflections were observed in the X-ray patterns of native and reconstituted nucleohistones, but not in the X-ray diffraction patterns of DNA or histones by themselves. It was proposed that the chromatin fiber is organized into a regular supercoil of pitch 100-120 Å. This model, although widely accepted for some time, has now come under question. Olins and Olins (2) have observed regular spacings of chromatin particles (termed ν-bodies) in water-swollen nuclei centrifuged onto electron microscope grids. These particles are 60-80 Å in diameter and are joined by thin filaments 15 Å in diameter. These results have been confirmed and extended by other laboratories (3-5). Nuclease digestion studies also support a subunit or particulate structure for chromatin: Hewish and Burgoyne (6) and Burgoyne et al. (7) have found that an endogenous Ca^{++}-Mg^{++} -activated endonuclease of rat and mouse liver nuclei cleaves chromatin DNA at regular intervals; fragments of DNA at integral multiples of about 200 base pairs were observed. Many workers have now studied the digestion of isolated chromatin with exogenous nucleases (8-14), and the results are generally consistent with the subunit or "beads-on-a-string" model of chromatin structure (2,15). Furthermore, isolated particles from nuclease-treated or sonicated chromatin resemble ν-bodies in the electron microscope (16-18).

Recent evidence obtained from neutron scattering of chromatin (19) suggests that the 110 Å reflection seen in the X-ray pattern does not arise from a DNA repeat, but rather from a regular spacing of a protein core. Baldwin et al. (19) and van Holde et al. (20) propose that chromatin DNA is wound about the exterior of the protein core. Noll's

finding of nuclease-sensitive sites every 10 base pairs along the DNA in chromatin appears to support this notion (21).

The work on chromatin structure cited above has been carried out with whole chromatin, unfractionated with respect to transcriptional activity. Since only a minor portion of the DNA in any given cell type is transcriptionally active, the properties of unfractionated chromatin primarily reflect the structure of inactive regions. We are interested in whether the template-active fraction of chromatin is organized in a structure similar to inactive regions or whether it is in a different conformation. Previous work from this laboratory has shown that it is possible to separate chromatin into active and inactive fractions (22-24). Transcriptionally-active chromatin appears to be preferentially attacked by the endonuclease DNase II; separation of the chromatin fractions is accomplished by centrifugation and selective precipitation of inactive chromatin with $MgCl_2$. The active fraction differs from whole chromatin or inactive chromatin in that it possesses a decreased histone to DNA ratio, lacks histone I, and has an increased nonhistone to DNA ratio (24). The active fraction comprises a subset of both single copy and repetitive DNA sequences and is enriched five-fold in sequences complementary to cellular RNA (24). In this communication we report that both active and inactive chromatin fractions contain nuclease-resistant particles; however, the nuclease-resistant structures of inactive chromatin are DNA-histone complexes while the nuclease-resistant particles of active chromatin are DNA-RNA-protein complexes made up largely of nonhistone chromosomal proteins.

METHODS

Chromatin fractionation. Chromatin was prepared from rat liver by the method of Marushige and Bonner (25). The chromatin pellet after sucrose gradient centrifugation was washed once with 10 mM Tris-HCl (pH 8) and dialyzed overnight at 4° against 200 volumes of 25 mM sodium acetate (pH 6.6). Fractionation was carried out as diagrammed in Fig. 1; details of this method have been published previously (24).

Preparation of chromatin subunits. Nuclease-resistant particles from rat-liver chromatin were prepared as follows: DNase II was added to 10 units per A_{260} unit of chromatin in 25 mM sodium acetate (pH 6.6). Digestion was carried out at 24° and was terminated after 90 min by raising the pH to 7.5 with 0.1 M Tris-HCl (pH 11). Nuclease-resistant subunits from chromatin fraction P1 were prepared by homogenizing the pellet fraction in 25 mM sodium acetate (pH 6.6) and redigesting with DNase as described above for whole chromatin. Undigested chromatin (about 20% of the input DNA) was removed by centrifugation at 27,000 g for 10-15 min. The supernatant was layered on isokinetic sucrose gradients in SW25.1 cellulose nitrate tubes. The gradients were formed according to Noll (26); the parameters were C_{TOP} = 15% (w/v), C_{RES} = 34.2% (w/v), and V_{MIX} = 31.4 ml. All solutions contained 10 mM Tris-HCl (pH 8). Centrifugation was at 25,000 rpm for 36-42 h. Gradients were analyzed with an ISCO UV Analyzer and chart recorder. Fractions from these gradients were rerun on a second isokinetic sucrose gradient. Sucrose was removed from the fractions by dialysis against 10 mM Tris-HCl (pH 8) and the sample was concentrated with an Amicon Minicon device prior to layering the sample on the gradient. The parameters for the

second isokinetic sucrose gradient were C_{TOP} = 5.1% (w/v), C_{RES} = 31.4% w/v, and V_{MIX} = 9.4 ml. All solutions contained 10 mM Tris-HCl (pH 8). Centrifugation was in the SW41 rotor at 39,000 rpm at 4° for 16.5 h. The gradients were analyzed spectrophotometrically as described above. Subunits were also prepared from chromatin devoid of histone I. Removal of this histone was accomplished by extraction of Virtis-sheared chromatin (45 v, 90 sec) with 0.5 M NaCl at 4°. The resultant nucleohistone was pelleted by centrifugation in the Ti 50 rotor at 50,000 rpm for 18 h. The pellet was homogenized in 10 mM Tris-HCl (pH 8) and treated in the same manner as whole or P1 chromatin.

Redigestion of Chromatin Fraction S2. Chromatin of fraction S2 was digested with nuclease in three different ways: the DNase II present in fraction S2 was reactivated by adding EDTA to 20 mM and lowering the pH to 6.4 with dilute HCl. Alternatively, aliquots of chromatin fraction S2 were dialyzed against either 25 mM sodium acetate (pH 6.6) or 5 mM sodium phosphate (pH 6.7) containing 2.5×10^{-4} M $CaCl_2$ and 2.5×10^{-4} M $MgCl_2$. DNase II was added to the chromatin in sodium acetate buffer to 10 units per A_{260} unit of chromatin; staphylococcal nuclease was added to the chromatin in sodium phosphate buffer to 50 units per ml. Reactions were carried out at 24°. Aliquots were taken at various times to test for the production of trichloroacetic acid-soluble material (measured by absorbance of the supernatant at 260 nm after centrifugation at 27,000 g at 4° for 15 min).

Sucrose Gradient Analysis of Fraction S2 Chromatin. Chromatin was prepared and fractionated as described above. The active fraction S2 was concentrated on an Amicon Minicon device, layered on a 5-24% isokinetic

sucrose gradient, and centrifuged in the SW41 rotor at 39,000 rpm for 17.5 h at 4°. The parameters for the gradients were C_{TOP} = 5.1% (w/v), C_{RES} = 31.4% (w/v), and V_{MIX} = 9.4 ml (26). All solutions contained 10 mM Tris-HCl (pH 8).

DNA Size Estimation. Single-strand DNA lengths were estimated by velocity sedimentation in alkaline sucrose gradients. The parameters for the isokinetic gradients were C_{TOP} = 15.9% (w/v), C_{RES} = 38.9% (w/v), and V_{MIX} = 6.1 ml (26). All solutions contained 0.1 N NaOH. Chromatin samples were suspended in 0.1 N NaOH, 2% sodium dodecyl sulfate, 2 M urea, and 100-200 µl aliquots were layered on each gradient. Centrifugation was in the SW50.1 rotor at 48,000 rpm for 16 h at 20°. DNA molecular weights were determined relative to a standard sized by electron microscopy (320 nucleotide-long, 5.4 S, calf thymus DNA; a gift of Ms. M. Chamberlin). Double-strand lengths were determined in the analytical ultracentrifuge (27).

Optical Thermal Denaturation Profiles were obtained with a Gilford 2400 Spectrophotometer. All samples were dialyzed against 0.2 mM EDTA (pH 8) prior to melting.

Analysis of Chromatin Composition. Histone and nonhistone protein content was determined as described (28). Protein was analyzed by sodium dodecyl sulfate-disc gel electrophoresis (29) and by acid-urea gel electrophoresis (30). DNA and RNA were determined by the methods of Schmidt and Tannhauser (31).

Enzymes. DNase II (E.C. 3.1.4.6) and micrococcal nuclease (E.C. 3.1.4.7) from staphylococcus were purchased from the Worthington Biochemical Corp.

RESULTS

Chromatin Fractionation. DNase II preferentially attacks a minor portion of chromatin DNA; the proportion of DNA in this fraction varies depending upon the source of the chromatin, but corresponds quite closely to the measured template activity of the particular chromatin (23). The fractionation scheme used herein is diagrammed in Fig. 1. After 5 min exposure to DNase II, 15% of rat-liver chromatin DNA remains soluble after centrifugation (fraction S1). About 11% of the total DNA is Mg^{++}-soluble and is found in fraction S2. This DNA comprises a subset of whole genomal DNA sequences and is enriched five-fold in transcriptionally-active sequences (24). The DNA has a double-strand length of about 700 base pairs and a single-strand length of 200-600 nucleotides (range of observed values). About 1-3% of this DNA is acid soluble. After 30 min exposure to DNase II, nearly 80% of the chromatin is found in fraction S1, and 20-24% is found in fraction S2. The S2 DNA averages 50 nucleotides, but half of this DNA is acid soluble. A more detailed description of the kinetics of DNase II digestion will appear elsewhere (Gottesfeld et al., in preparation).

Table I lists some of the properties of the chromatin fractions: the composition of fraction P1 is similar to that of unfractionated rat-liver chromatin (24-25). Fraction S2, however, is enriched in RNA and nonhistone protein and has a reduced content of histone protein. As re-

Fig. 1. Fractionation scheme. The yields of DNA in each fraction are the mean and standard deviation for 11 determinations.

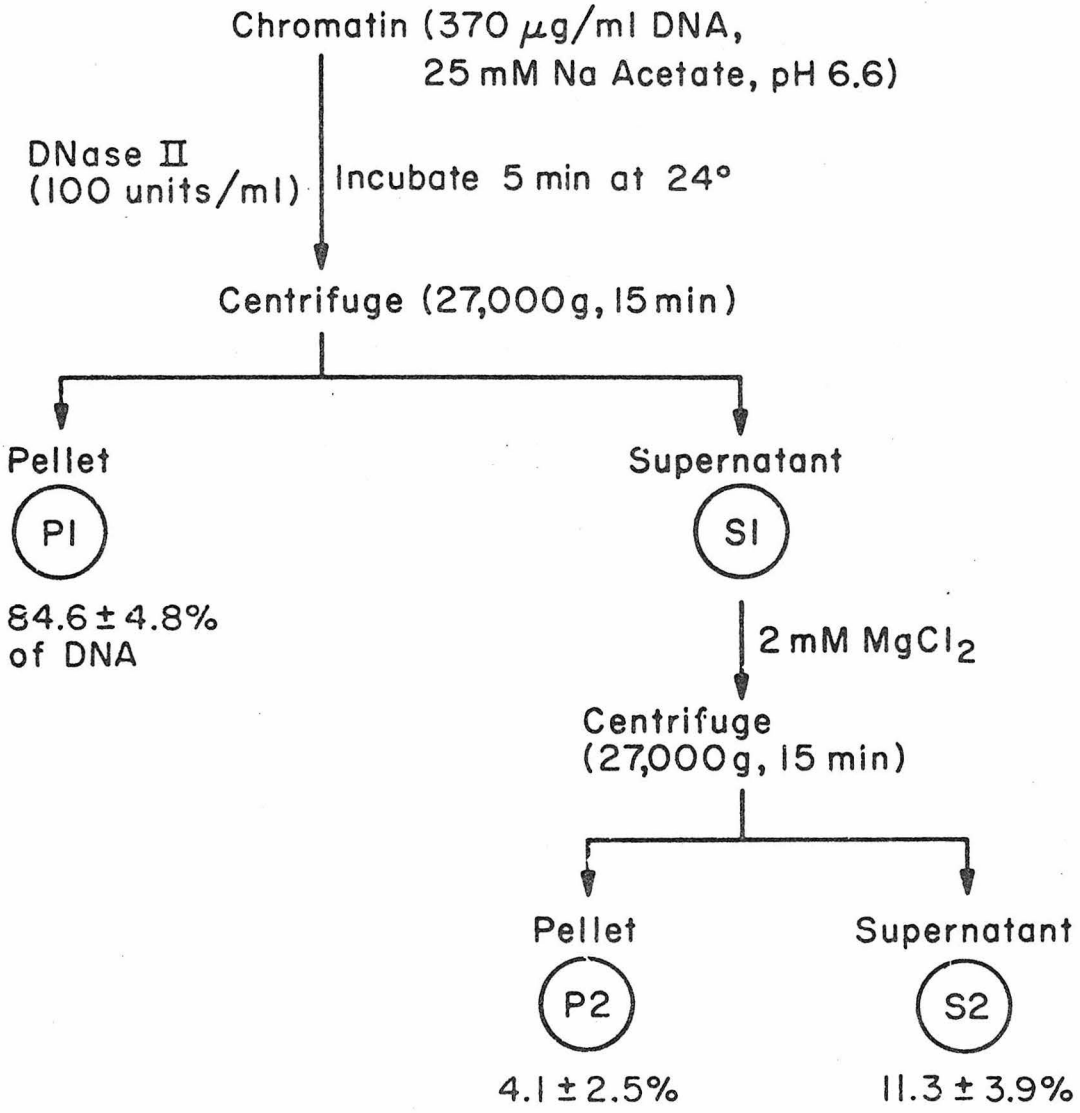

Chromatin (370 μg/ml DNA,
25 mM Na Acetate, pH 6.6)
DNase II
(100 units/ml)
Incubate 5 min at 24°
Centrifuge (27,000g, 15 min)
Pellet
P1
84.6 ± 4.8%
of DNA
Supernatant
S1
2 mM MgCl2
Centrifuge
(27,000g, 15 min)
Pellet
P2
4.1 ± 2.5%
Supernatant
S2
11.3 ± 3.9%

Table I. Chemical Composition of Chromatin Fractions.

Sample	Composition Relative to DNA (w/w)				
	DNA	RNA	Total Protein	Histone Protein*	Nonhistone Protein
P1 Chromatin	1.00	0.05	1.73	1.15	0.58
"12 S" Subunits	1.00	-	1.01	0.96	<0.05
S2 Chromatin	1.00	0.25	2.21	0.61	1.60
S2 Subfractions					
3-5S	1.00	-	0.88±0.06	0.24	0.60
14.9±0.8S(16)+	1.00	0.3-0.4	2.94±0.06	0.72	1.35
20.2±0.7S(16)+	1.00	0.3-0.7		0.54	3.2

Table I. Footnote.

*Chromatin was separated into acid-soluble (histone) and acid-insoluble (nonhistone) protein (28). For P1, "12S" and total S2 chromatin, all acid soluble protein indicated is histone; however, we do not know what proportion of acid soluble protein of the S2 subfractions is histone protein.

+Sedimentation values ± S.D. determined from 16 gradients equivalent to those in Fig. 3.

ported previously (24), fraction S2 lacks histone I.

The melting characteristics of the chromatin fractions are presented in Table II. The mean temperature of thermal denaturation (T_m) of the DNA in P1 chromatin is 69°; however, several transitions can be recognized in a derivative plot of the melting data. Li and Bonner (32) have investigated the melting of pea bud nucleohistone and have assigned the transition at 42° to regions of free DNA, the transitions at 52-60° to DNA complexed with nonhistone protein, and the transitions at 66 and 81° to DNA complexed with histone protein. Table II lists the fraction of total hyperchromicity in each of five melting transitions observed in rat-liver chromatin. About two-thirds of the hyperchromicity of P1 chromatin DNA comes from melting transitions IV and V, the histone-induced transitions. In contrast, S2 chromatin DNA has a T_m of 55° and about two-thirds of the hyperchromicity is due to nonhistone-induced DNA melting and free DNA melting transitions. This result is consistent with the chemical compositions of these chromatin fractions. It should be noted that S2 chromatin exhibits a lower final hyperchromicity than either DNA or P1 chromatin (Table II). The range of hyperchromicities observed for S2 chromatin is 7-16% with a mean and standard deviation of 11.4 ± 3.5% (6 determinations). This reduced hyperchromicity cannot be due solely to the RNA content of this fraction; perhaps much of the DNA in S2 chromatin is unpaired or is in DNA-RNA hybrids. The source of the decreased hyperchromicity is removed by the combination of pronase and ribonuclease treatment; purified DNA from fraction S2 exhibits 70-75% of native hyperchromicity.

Table II. Melting Properties of Chromatin Fractions.

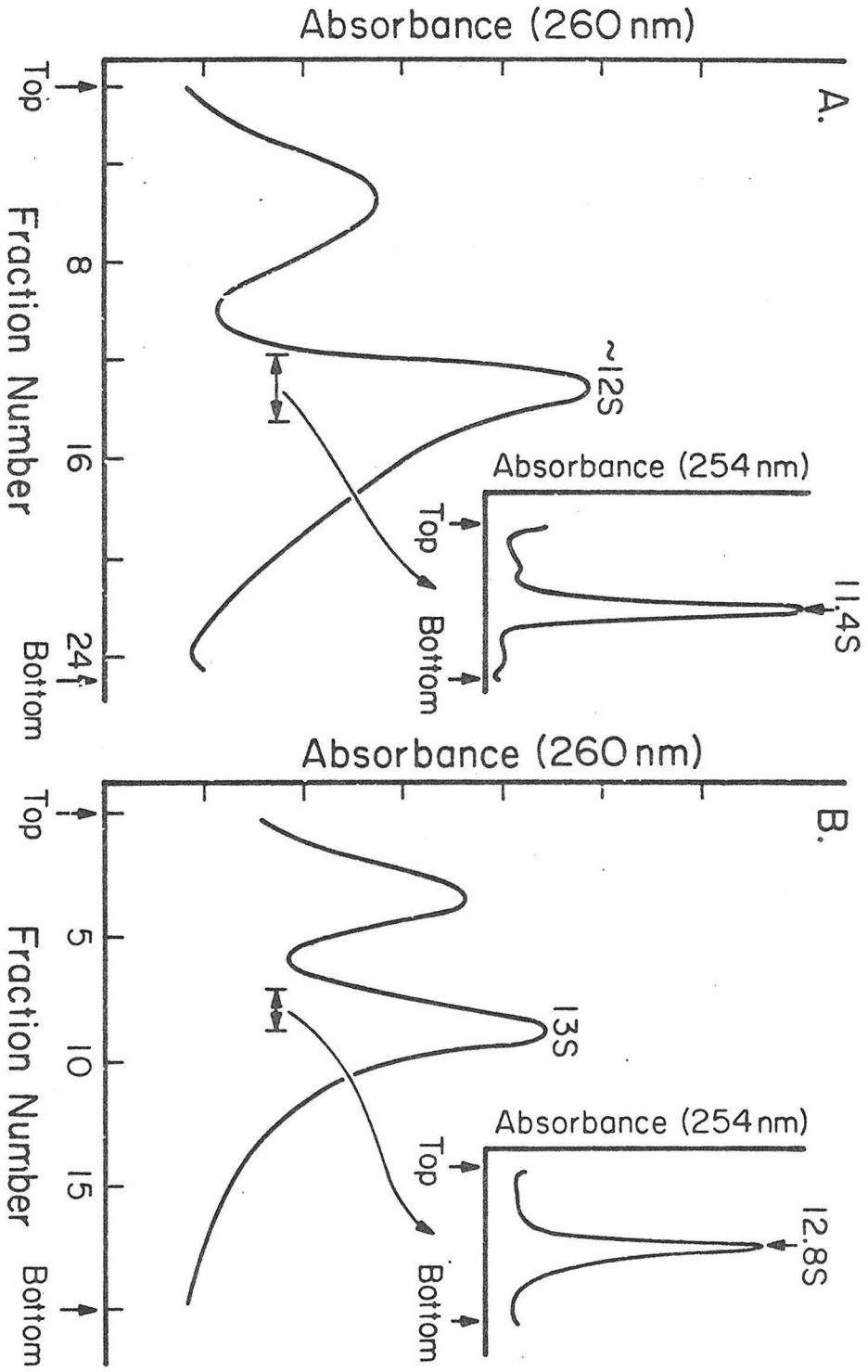
Sample	Average Melting Temperature (T_m) (°C)	Hyperchromicity (%)	Fraction of Hyperchromicity in Melting Transition*					
			I (42°)	II (52°)	III (61°)	IV (67°)	V (76°)	
DNA	42	35	1.0	0	0	0	0	
S2 Chromatin	55	11	0.18	0.21	0.21	0.25	0.15	
P1 Chromatin	69	33	0.03	0.11	0.20	0.31	0.34	
							(76°)	(81°)
"12S" Subunits	77	35	0	0	0	0	0.58	0.42

*Derivative plots of the data were calculated according to Li and Bonner (32) and the relative area under each transition was determined.

Subunit Structure of Chromatin. Rat-liver chromatin and fraction P1 chromatin have been digested with DNase II for extended periods of time (90 min), and the resulting soluble chromatin analyzed by centrifugation in isokinetic sucrose gradients (Fig. 2). About 40% of the input DNA sediments extremely slowly; the bulk of this DNA is acid soluble and hence has been reduced to oligonucleotides by the nuclease. Fig. 2 presents data for whole chromatin depleted of histone I (Fig. 2A) and for P1 chromatin (Fig. 2B). Most of the chromatin sediments at about 11-13S, with some material sedimenting more rapidly. The properties of nuclease-resistant particles from unfractionated chromatin have been described in detail elsewhere (8-14, 16, 18). We find similar properties for the particles from P1 chromatin: the particles are composed of equal amounts of protein and DNA (by weight), and the protein complement is almost entirely histone (Table I). Subunits from native chromatin sediment slightly more rapidly than subunits from histone I-depleted chromatin (Fig. 2). The DNA of subunits isolated from native chromatin is 211 ± 9 nucleotides in length while subunits from histone I-depleted chromatin contain DNA 166 ± 16 nucleotides in length. These values were estimated by sedimentation velocity measurements under alkaline conditions. The nuclease-resistant particles resemble ν-bodies (2) in the electron microscope (18).

The DNA in the nuclease-resistant particles is highly stabilized against thermal denaturation; melting transitions at 76° and 81° can be recognized in the derivative plot of the data (Table II). The overall T_m is 77°, about 35° above the T_m of deproteinized DNA in the same solvent.

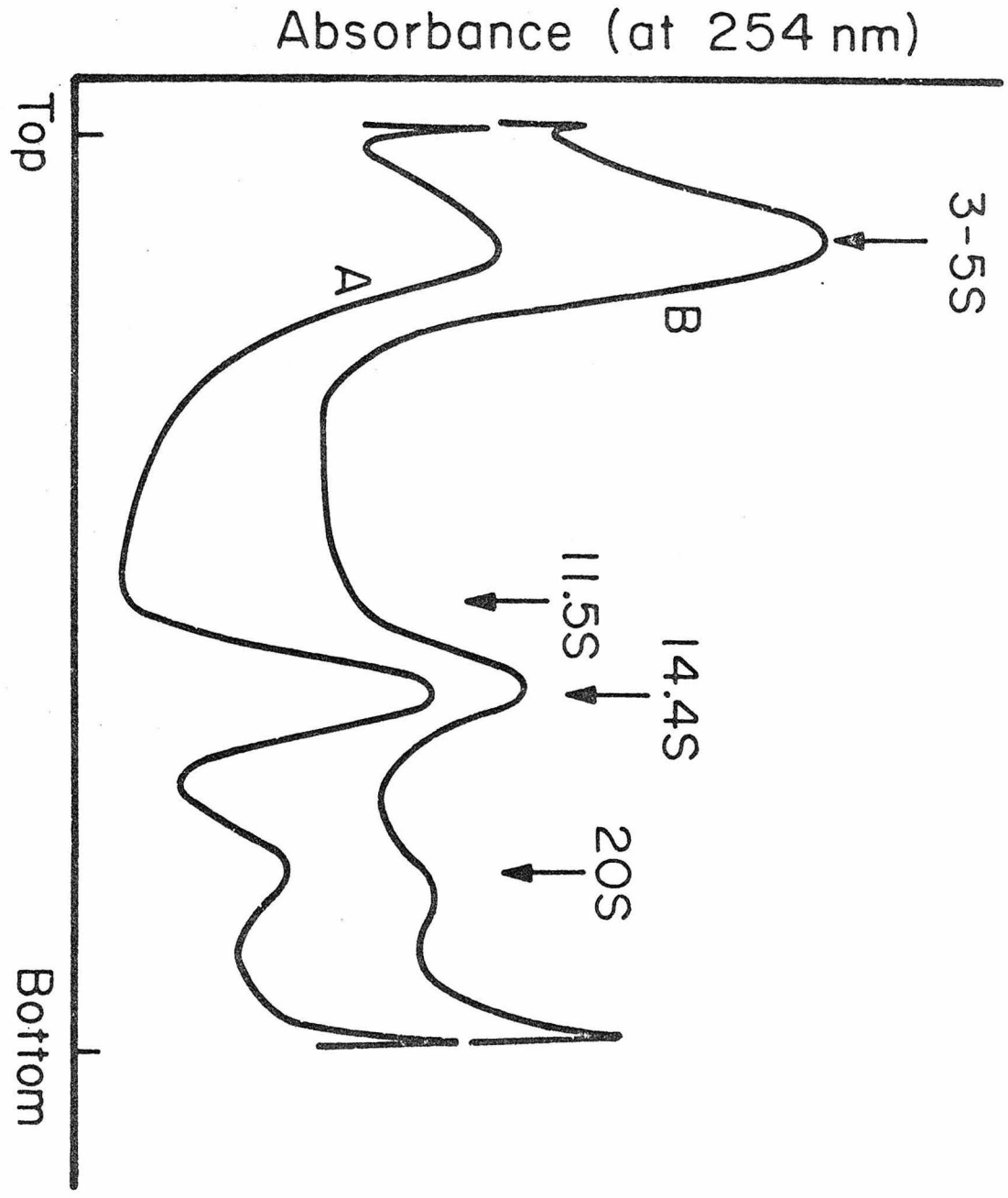
Fig. 2. Sucrose gradient sedimentation of DNase II-treated chromatin samples. A. Histone I-depleted chromatin was digested as described in Methods; the soluble chromatin was layered on isokinetic gradients and centrifuged for 42 hr in the SW25.1 rotor. Fractions were pooled as indicated and rerun on 5-24% isokinetic gradients (inset). B. Fraction P1 chromatin was digested and soluble chromatin was centrifuged for 36 hr. Fractions were pooled as indicated and rerun (inset).


Absorbance (260 nm)
A.
Top
Fraction Number
8
16
24
Bottom
~12S
Absorbance (254 nm)
Top
Bottom
11.4S
Absorbance (260 nm)
B.
Top
5
10
15
Fraction Number
Bottom
13S
Absorbance (254 nm)
Top
Bottom
12.8S

Subunit Structure of Active Chromatin. We now ask whether nuclease-resistant particles occur in transcriptionally active regions of chromatin. Chromatin from rat liver was treated with DNase II for 5 min, fractionated as before (Fig. 1), and S2 material analyzed on isokinetic sucrose gradients (Fig. 3, lower curve). About half of the UV-absorbing material applied to the gradient sediments at 3-5S; greater than 90% of this material is acid precipitable after the 5 min nuclease treatment. Two more rapidly sedimenting peaks are seen in the gradient of S2 chromatin: one at 14-15S and another at 19-21S. These gradients were calculated for particles of density 1.44 g/cm^3, and so the observed sedimentation coefficients could be in error if the particle densities are very different from 1.4-1.5 g/cm^3. About 6% of the input nucleic acid pelleted during the centrifugation.

It was found previously that histone I-deficient subunits sediment at 11.5 ± 0.3S (Fig. 2A). Since chromatin fraction S2 lacks histone I, we would expect that if this fraction contains nuclease-resistant "ν-body" structures, these particles would sediment at 11-12S. No material was seen at this S value in fraction S2 chromatin (Fig. 3); however, fraction S2 produced by a 5 min DNase digestion might contain these particles in the form of dimers and higher multimers (>14S). Therefore, fraction S2 was redigested with DNase and analyzed as before (Fig. 3, upper curve). Redigestion was accomplished by reactivating the DNase present in fraction S2 by the addition of EDTA (pH 6.4, see METHODS). The EDTA presumably acts by chelating inhibiting Mg^{++} ions. Reincubation of S2 chromatin was carried out at 24° for 20-120 min. Upon analysis in isokinetic sucrose gradients, no significant changes were observed in the >10S region of the gradients.

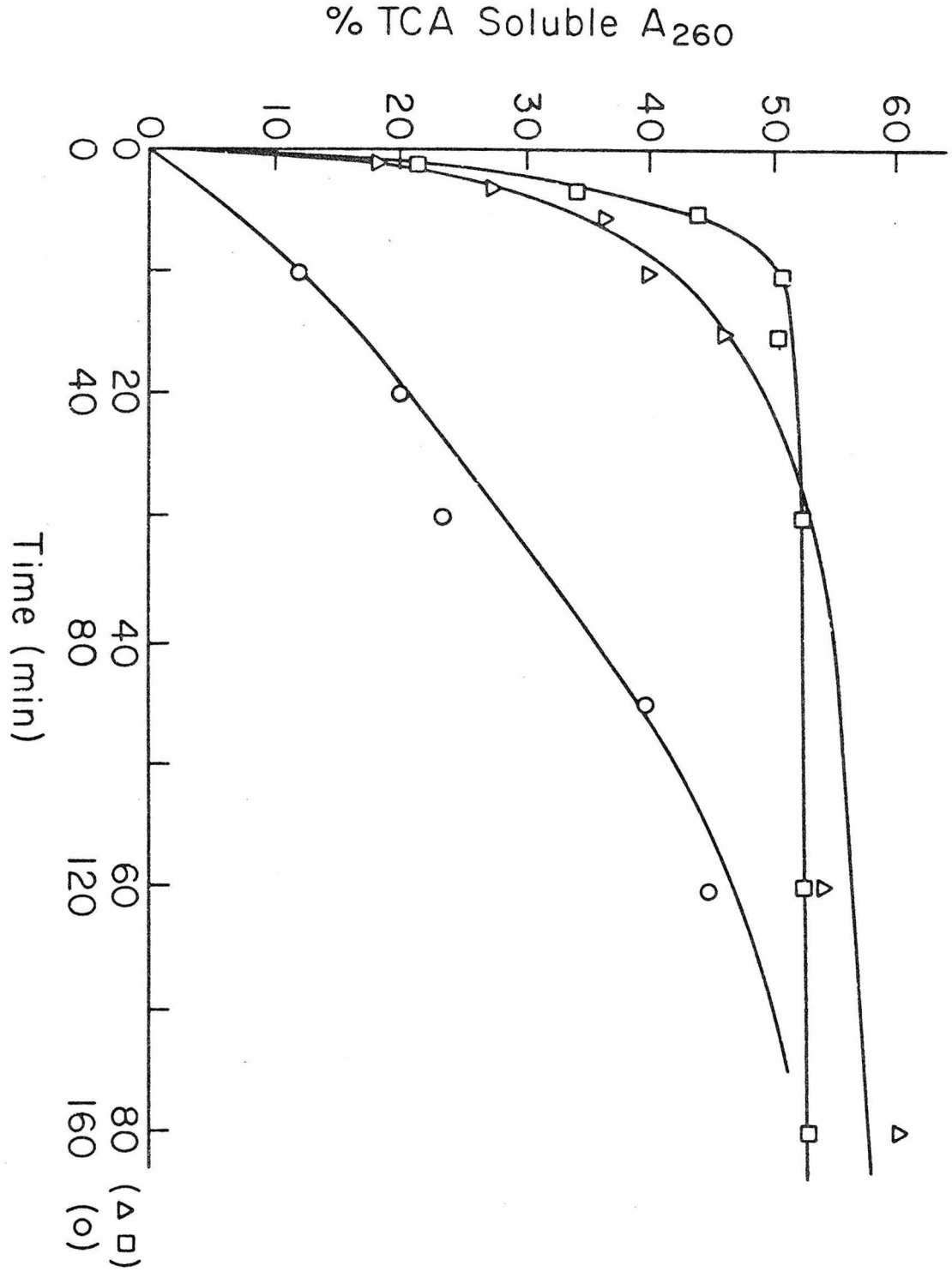
Fig. 3. Sucrose gradient sedimentation of template-active fraction S2 chromatin. Curve A: Chromatin was fractionated (Fig. 1) and S2 material centrifuged for 17.5 hr at 39,000 rpm in an isokinetic gradient. Curve B: Fraction S2 was isolated and DNase II reactivated by the addition of 20 mM EDTA (pH 6.4). Incubation was for 1 hr at 24°. The reaction was terminated by raising the pH to 8 with 0.1 M Tris·HCl (pH 11), and the sample was centrifuged for 17.5 hr at 39,000 rpm in an isokinetic sucrose gradient.


Absorbance (at 254 nm)
Top
Bottom
A
B
3-5S
11.5S
14.4S
20S

On redigestion of S2 chromatin, it was found that 50-60% of the UV-absorbing material became acid-soluble (Fig. 4). Redigestion has been performed in three ways: reactivation of DNase II; addition of fresh DNase II under standard conditions of enzyme treatment; and addition of staphylococcal nuclease. In all cases, 50-60% of the input S2 chromatin A_{260} became acid soluble. When the reaction approached completion (50% digestion), the solutions became turbid; this was observed with both nucleases. The nuclease-resistant chromatin DNA was found to have a weight-average single-strand length of 120 nucleotides. These observations on S2 chromatin are similar to those of Clark and Felsenfeld (8-9) with unfractionated chromatin.

The chromatin species at 15 and 20S both exhibit T_m's of 55° (0.2mM EDTA, pH 8) and reduced hyperchromicities relative to DNA or P1 chromatin. The material at 3-5S exhibits the same T_m as protein-free DNA, 42°. The chemical compositions of the subfractions of S2 chromatin have been determined (Table I). The material at 15 and 20S is enriched in both RNA and nonhistone chromosomal proteins.

Fig. 4. Kinetics of digestion of chromatin fraction S2. Chromatin was fractionated as described (Fig. 1). S2 chromatin was incubated with nucleases in three ways: reactivation of DNase II (o); addition of fresh DNase II (Δ); addition of staphylococcal nuclease (□). Aliquots were taken at various times to test for the production of trichloroacetic acid-soluble material (measured by absorbance at 260 nm). The TCA concentration was 7% (w/v).


% TCA Soluble A_{260}
0
10
20
30
40
50
60
Time (min)
0
20
40
80
120
160
(Δ □)
(o)

DISCUSSION

It appears that the template-active fraction (S2) is organized in a fashion similar to that of inactive chromatin; that is, the active fraction is composed of regions of nuclease-sensitive and nuclease-resistant DNA. However, nuclease-resistant segments in transcriptionally inactive chromatin are due to histone-DNA interactions (the ν-bodies), while the nuclease-resistant portions of active chromatin arise from the interaction of DNA with both histone and nonhistone chromosomal proteins. Thermal denaturation of S2 chromatin (Table II) shows that S2 DNA is complexed primarily with the nonhistone proteins (32). These results shed new light on the findings of Felsenfeld's laboratory (8-9,33). These investigators have reported that portions of the globin gene are found in both nuclease-sensitive ("open") and nuclease-resistant ("closed") regions of reticulocyte chromatin. The present communication suggests that Felsenfeld's method of isolation of "open" and "closed" segments of chromatin (33) should not discriminate between transcriptionally active and inactive chromatin regions, in agreement with the globin result (33). Felsenfeld's data indicates that regions of the globin genes are always covered by protein (33) but makes no distinction between histone or nonhistone protein. From the present data, we speculate that this protein might be nonhistone.

The distribution of nuclease-resistant complexes in template-active chromatin parallels that of inactive chromatin; however, thermal denaturation and circular dichroism studies (34,35) suggest that active chromatin is in a more extended, DNA-like conformation than inactive chromatin. Nuclease-resistant particles from whole chromatin exhibit a nonconservative circular dichroism spectrum, suggesting that the DNA may be in a conformation different from that of the B-form in aqueous solution (11). Protein-induced folding of B-form DNA might also account for the observed circular dichroism spectrum. The electron microscope has revealed differences in the structure of transcriptionally active and inactive regions of chromatin. Ribosomal genes in the act of transcription are the length of their transcription product (pre-rRNA) (36). On the other hand, DNA complexed with histones in the ν-body configuration is one-seventh the length of deproteinized DNA (4). The basic fiber diameter of inactive chromatin is 100 Å; however, active chromatin has a fiber diameter of about 30 Å (37-38). Thus both physical chemical and E.M. studies suggest that the DNA of active chromatin is more extended than the DNA of inactive chromatin. It is likely that this is why active chromatin is more susceptible to nuclease; this differential sensitivity to nuclease forms the basis of our fractionation procedure (Fig. I). We have found that isolated ν-bodies and ν-body containing chromatin is precipitated by divalent cations (Gottesfeld et al., unpublished), while active chromatin remains soluble. We propose that the ν-body is both the basic unit of structure and the physical mechanism of gene repression in the eukaryotic chromosome. The subunit structure of active chromatin appears to be different from that of inactive chromatin. Further work on the active fraction should yield

much valuable information about the structure and mechanisms of the eukaryotic gene regulatory apparatus.

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VI. BIOPHYSICAL STUDIES ON THE MECHANISM OF QUINACRINE STAINING OF CHROMOSOMES

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Biophysical Studies on the Mechanism of Quinacrine Staining of Chromosomes†

J. M. Gottesfeld,* J. Bonner, G. K. Radda, and I. O. Walker

ABSTRACT: The fluorescence of quinacrine was measured in solution in the presence of interphase chromosomal material (chromatin) and in the presence of chromatin which had been fractionated into extended and condensed regions (euchromatin and heterochromatin). Quinacrine fluorescence is quenched most effectively by the euchromatin fraction, intermediately by unfractionated chromatin, and least effectively by the heterochromatin fractions. These differences are abolished when the fluorescence of quinacrine is measured in the presence of DNA isolated from chromatin and the chromatin fractions. Spectrophotometric titrations indicate that the association constants for quinacrine binding to the various chromatin fractions differ by only a factor of two, and that the number of sites per nucleotide available for quinacrine binding at saturation are nearly identical for all fractions. Circular dichroism spectroscopy suggested that the conformation of the DNA in the euchromatin fraction is most like that of protein-free DNA in aqueous solution ("B"-form DNA) while the DNA in the heterochromatin fractions is partially in the "C" conformation. These results suggest that protein-DNA interactions in chromatin are responsible for the fluorescence patterns observed and that chromosome banding with quinacrine might arise from differences in protein-DNA interactions (and DNA conformation) along the chromatids of metaphase chromosomes.

Caspersson *et al.* (1968) and others have shown that the fluorescent dye quinacrine mustard stains specific regions of chromosomes with a very brilliant intensity, leaving other areas of chromosomes relatively dark. It was originally thought that the linear differentiation of chromosomes into fluorescent bands and poorly staining interband regions was due to the specific alkylation of guanine residues by the mustard function of the dye. The finding that quinacrine itself produces identical banding patterns suggested that alkylation of guanine residues was not the primary mechanism of fluorescence staining of chromosomes (Caspersson *et al.*, 1969). Ellison and Barr (1972) have suggested that enhancement of quinacrine fluorescence might be a function of base ratio, with (A + T)-rich regions fluorescing brightly. Weisblum and de Haseth (1972) and Pachmann and Rigler (1972) have investigated quinacrine fluorescence *in vitro* with a series of natural and synthetic polynucleotides, and found that A·T base pairs are responsible for fluorescence enhancement. Guanine residues were shown to give rise to quenching of quinacrine fluorescence. These data, and several other lines of evidence (Schreck *et al.*, 1973; Lomholt and Mohr, 1971), suggested that the fluorescent bands observed with quinacrine-stained chromosomes are indeed (A + T) rich.

This investigation was undertaken to determine whether quinacrine flourescence in the presence of isolated chromosomal material is due solely to intrachromosomal differences in DNA base composition, or whether DNA-protein interactions in chromatin play a role in producing banding patterns. Chromatin has been fractionated into extended and condensed regions (euchromatin and heterochromatin) (Billing and Bonner, 1972; Bonner *et al.*, 1974; Gottesfeld *et al.*, 1974). It has been suggested that the extended chromatin fraction corresponds to

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TABLE I: Distribution of DNA in the Chromatin Fractions.

Sample	% of Total DNA	
	5-min DNase Exposure	15-min DNase Exposure
Rat chromatin		
Euchromatin (S2)	11.3 ± 3.9[a]	25.0 ± 3.0[b]
Heterochromatin (P1)	84.6 ± 4.8	23.9 ± 2.9
Heterochromatin (P2)	4.1 ± 2.5	50.6 ± 3.3
Drosophila chromatin		
Euchromatin (S2)		25.6 ± 3.2[c]
Heterochromatin (P1)		28.2 ± 1.9
Heterochromatin (P2)		45.4 ± 5.8

[a] Mean of 11 determinations ± SD. [b] Mean of 9 determinations ± SD. [c] Mean of 3 determinations ± SD.

the template active portion of chromatin *in vivo* and that the heterochromatin fractions correspond to the repressed or template inactive portion of chromatin *in vivo* (Bonner *et al.*, 1973, 1974; Gottesfeld *et al.*, 1974). Fluorescence and binding data have been obtained for the interaction of quinacrine with chromatin and the chromatin fractions. Significant differences in the quantum yield of quinacrine bound to these fractions were found; these differences were found to be due to protein–DNA interactions and not differences in DNA base composition. In addition, circular dichroism studies of the chromatin fractions have yielded information on the protein-induced conformational alteration of DNA in these fractions.

Materials and Methods

Preparation of Chromatin. Chromatin from rat liver was prepared by the method of Marushige and Bonner (1966). *Drosophila* embryo chromatin was a gift of Dr. S. C. R. Elgin (Elgin and Hood, 1973). The crude chromatin from both rat and *Drosophila* was purified by centrifugation through 1.7 M sucrose as described by Marushige and Bonner (1966). The sucrose pellet was homogenized in 0.01 M Tris-Cl (pH 8) and centrifuged at 27,500*g* for 20 min.

Fractionation of Chromatin. Purified chromatin was homogenized with a motor-driven glass-Teflon homogenizer (20 strokes at 10 strokes per minute) in approximately 20–30 ml of 0.01 M Tris-Cl (pH 8). The homogenate was dialyzed overnight *vs.* 6 l. of 0.025 M sodium acetate (pH 6.6). After dialysis, the dialysate was homogenized as before with the motor-driven glass-Teflon homogenizer. The volume of the chromatin solution was adjusted to give 10 A_{260} units/ml when the absorbance of an aliquot (100 μl) was measured in 0.9 N NaOH. DNase II (Worthington, HDAC) was added to 10 units of enzyme/A_{260} unit of chromatin (100 units of enzyme/ml). The digestion was allowed to proceed for 5–15 min at ambient temperature (24°). At the end of the incubation period, the pH of the chromatin suspension was brought to 7.5 with 0.02 M Tris-Cl (pH 11). The chromatin suspension was cooled on ice and then centrifuged at 27,500*g* for 15 min at 0–4°. To the supernatant, 0.2 M $MgCl_2$ was added dropwise to give a final concentration of 0.002 M. After 30-min stirring at 0–4°, the solution was centrifuged again at 27,500*g* for 15 min.

The final supernatant from above is termed S2 (extended euchromatin). A 5-min nuclease treatment of rat liver chromatin yields 10–15% of the total chromatin nucleic acid in S2. The DNA prepared from the S2 chromatin fraction (5-min DNase treatment) is double stranded and 700 base pairs in length (number-average length). Longer exposure to DNase II yields up to 25% of the chromatin DNA in S2. When prepared from *Drosophila* embryo chromatin, the euchromatin fraction also contains 25% of the total chromatin DNA (15-min digestion). Table I lists the amounts of nucleic acid found in the fractions obtained from both rat liver and *Drosophila* chromatin.

The pellet heterochromatin fractions are termed P1 (first pellet) and P2 (second pellet). Gottesfeld and Bonner (1974) have shown that the P1 fraction from rat chromatin is relatively inaccessible to enzyme attack. Thus the DNA in this fraction is likely to be more highly compacted and may correspond to the constitutive or centric heterochromatin in metaphase chromosomes. Measurement of template activity and DNA–RNA hybridization studies (Gottesfeld *et al.*, 1974) show that the pellet or heterochromatin fractions correspond to the template inactive fraction of interphase chromatin. Table I indicates that very little nucleic acid is found in the second pellet fraction (P2) after a 5-min enzyme treatment. Bonner *et al.* (1973, 1974) have given the chemical compositions of the fractions obtained from rat chromatin. Evidence for the validity of this fractionation procedure is also presented in Bonner *et al.* (1973, 1974) and in Gottesfeld *et al.* (1974).

Preparation of DNA. DNA was prepared from chromatin and the chromatin fractions in the following manner. The chromatin preparations were first extracted with phenol (saturated with 0.01 M Tris-Cl, pH 8) until no visible material remained at the interface. The aqueous phase was extracted repeatedly with an equal volume of chloroform–isoamyl alcohol (24:1, v/v). The aqueous phase was then dialyzed overnight *vs.* 0.01 M Tris-Cl (pH 8), containing 0.5 M NaCl. The dialysate was treated with ribonuclease (Worthington, RAF; preincubated at 80° for 10 min) at 50 μg/ml for 1 hr at 37°. After ribonuclease treatment, EDTA (pH 8) was added to 0.01 M and sodium dodecyl sulfate to 1% (w/v). Pronase (Calbiochem, CB; preincubated at 37° for 1 hr) was added to 50 μg/ml, and the mixture was incubated at 60° for 1–2 hr. After enzyme treatments, the DNA was phenol and chloroform–isoamyl alcohol extracted as before. The final DNA solution was dialyzed extensively *vs.* distilled water and then lyophilized.

Estimation of Nucleic Acid Concentrations. DNA concentrations were determined spectrophotometrically using an $\epsilon_{260\,nm}$ value of 6600. DNA concentrations of chromatin samples were estimated spectrophotometrically on solutions diluted with 1 N NaOH (final NaOH concentrations were 0.9–0.98 N). An $A_{260\,nm}$ reading of 1.0 corresponds to 37 μg/ml of chromatin DNA.

Fluorescence Measurements. Fluorescence spectroscopy was performed with a Hitachi Perkin-Elmer spectrofluorimeter Model MPF-2A. All measurements were made at 24–25°. Fluorescence of quinacrine in the presence of nucleic acid and nucleoprotein preparations was measured at a quinacrine concentration of 2.0×10^{-6} M. The buffers used in this study were 0.1 M sodium phosphate (pH 6.8–7.0) and 0.01 M Tris-Cl (pH 8). Fluorescence values reported herein are given relative to the fluorescence of quinacrine measured in the same solvent. Quinacrine was the dihydrate hydrochloride (mol wt 506.9) obtained from the Sigma Chemical Co., St. Louis, Mo. The excitation wavelength was 424 nm, and an emission spectrum was obtained for each sample. The excitation and emission slits were maintained at 6 nm.

To test the effect of chromosomal proteins on the fluorescence of a quinacrine–chromatin complex, Pronase was added to the mixture and the fluorescence output of the solution was monitored with time. To 0.5 ml of a solution containing rat

liver PI fraction heterochromatin (at 83 μg/ml of chromatin DNA) and quinacrine (at 2.0×10^{-6} M) in 0.01 M Tris-Cl (pH 8), 50 μl of a 200-μg/ml solution of Pronase (preincubated at 37° for 1 hr) was added at time zero. The excitation wavelength was 424 nm, and emission was monitored for 30 min at 495 nm. No significant instrument drift was observed with quinacrine alone measured at intervals over a 30-min period. Pronase (in the absence of nucleoprotein) had no effect on the fluorescence of quinacrine.

Measurement of Quinacrine Binding to DNA, Chromatin, and the Chromatin Fractions. Owing to the fact that quinacrine fluorescence is affected differently by A·T and G·C base pairs (Weisblum and de Haseth, 1972; Pachmann and Rigler, 1972), fluorescence titrations with natural DNAs cannot be used to estimate association constants or the number of sites per nucleotide available for dye binding. To study the interaction of quinacrine with DNA and chromatin quantitatively, we made use of the fact that the optical extinction coefficient of quinacrine is reduced on binding to DNA. Pachmann and Rigler (1972) have shown that both A·T- and G·C-containing polynucleotides cause a hypochromic shift in the absorption spectrum of quinacrine. Blake and Peacocke (1968) have presented a detailed treatment of the use of spectrophotometric titrations and the Scatchard plot in studying the binding of aminoacridines to nucleic acids. Briefly, if one plots r/C_F *vs.* r, where r is defined as the ratio of moles of bound dye (quinacrine) to moles of DNA nucleotides and C_F is the molar concentration of free dye, then a straight line is obtained for each class of binding sites present if the sites within each class are equivalent and independent of one another. The slope of the straight line is $-K_a$, the association constant, and the intercept on the abscissa is $\bar{n}$, the maximal number of moles of dye bound per mole of DNA nucleotide.

The molar extinction coefficient of bound quinacrine, $\epsilon_{420\ nm}{}^b$, was obtained experimentally for each DNA and nucleoprotein sample. At a constant concentration of quinacrine (4.4×10^{-5} M) in 0.01 M Tris-Cl (pH 8), the nucleic acid concentration was varied over the range of 1.0×10^{-5} to 3.0×10^{-4} M. The absorbancies of the quinacrine-DNA or quinacrine-nucleoprotein complexes were measured at 420 nm with a blank of DNA or nucleoprotein in the absence of quinacrine. This serves to correct for light scattering at high nucleoprotein concentrations. The absorbance at 420 nm was obtained from an absorption spectrum performed with a Cary Model 11 recording spectrophotometer. All absorption measurements were made at ambient temperature (24°). The value of $\epsilon_{420\ nm}{}^b$ was obtained from the absorption value at infinite nucleotide concentration. This latter value was obtained from a computer fit of a double-reciprocal plot of the experimental data by the method of least squares. Table II lists the extinction coefficients for free quinacrine and for quinacrine bound to various DNA and nucleoprotein preparations.

Data for the Scatchard plot were obtained from experiments where the concentration of quinacrine was varied over a 15-fold range at a fixed concentration of nucleic acid (DNA or chromatin). These experiments were performed as follows. To 3.0 ml of a solution containing DNA or nucleoprotein (ranging in concentration from 1.4 to 2.5×10^{-4} M in DNA nucleotides) in 0.01 M Tris-Cl (pH 8.0), 5- to 20-μl aliquots of a 5.0×10^{-3} M solution of quinacrine were added. After each addition of quinacrine an absorption spectrum was taken. A blank containing DNA or nucleoprotein in the absence of quinacrine was used to compensate for light scattering. The absorption spectrum of free quinacrine at each concentration (1.0–15×10^{-5} M) was also obtained. Beer's law was found to be obeyed over this range of quinacrine concentrations. The molar fraction of bound quinacrine was obtained from the absorbance readings as described by Blake and Peacocke (1968). To obtain K_a, the association constant, and $\bar{n}$, the maximal number of moles of quinacrine bound per mole of DNA nucleotides, a computer fit of the Scatchard plot by the least-squares method was performed.

TABLE II: Extinction Coefficients of Quinacrine (Free) and Quinacrine Bound to Rat Liver DNA, Chromatin, and Chromatin Fractions.

Sample[a]	$\epsilon_{420\ nm}$
Quinacrine, free	7.6×10^3
Quinacrine, bound to	
DNA	3.9×10^3
Chromatin	3.8×10^3
Euchromatin	3.9×10^3
Heterochromatin	4.1×10^3

[a] All measurements were made in 0.01 M Tris-Cl (pH 8).

Circular Dichroism Spectroscopy. Circular dichroism spectra were obtained with a Roussel-Jouan Dichrograph II and with a Durrum-Jasco ORD/UV-5. Spectra were recorded at room temperature with a sample cell of 1-cm path length. Spectra were scanned from 350 nm downward to lower wavelengths until the noise level became too high to record meaningful data (usually around 210 nm). CD spectra were obtained with samples at $A_{260\ nm}{}^{1\ cm}$ values of 0.8–1.5. Spectra are reported in terms of the difference in extinction coefficients for left and right circularly polarized light, respectively. $\epsilon_l - \epsilon_r$ is defined as $(A_l - A_r)/lc$, where A_l and A_r are the absorbancies for left and right circularly polarized light, respectively, l is the path length of the sample in centimeters, and c is the molar concentration of the sample. All spectra presented herein are reported in terms of molar concentrations of DNA nucleotides.

Thermal Denaturation. The mean temperature of thermal denaturation (T_m) of the DNA samples was determined with a Gilford spectrophotometer-multiple sample absorbance recorder equipped with a Haake circulating bath. The temperature of the bath was increased at a linear rate of about 0.5°/min. DNA samples were dialyzed against standard saline citrate (0.15 M NaCl-0.015 M trisodium citrate) prior to denaturation.

Determination of DNA Size. The size of the DNA prepared from chromatin and the chromatin fractions was determined by electron microscopy. The DNA samples were spread from aqueous ammonium acetate by the method of Davis *et al.* (1971).

Results

Quinacrine Fluorescence. FLUORESCENCE OF QUINACRINE ON BINDING TO DNA AND SYNTHETIC POLYNUCLEOTIDES. When excited at 424 nm, the fluorescence maximum of quinacrine in aqueous solution is 490–500 nm. The addition of naturally occurring DNAs of moderate A·T content (less than 60% A·T) or G·C-containing polynucleotides to a solution of quinacrine causes the quenching of fluorescence. On the other hand, (A + T)-rich DNAs and polynucleotides cause an enhancement of quinacrine fluorescence (Weisblum and de Haseth, 1972; Pachmann and Rigler, 1972). Neither quenching nor enhancement of fluorescence affects the shape or the position of the maximum of the quinacrine emission spectrum.

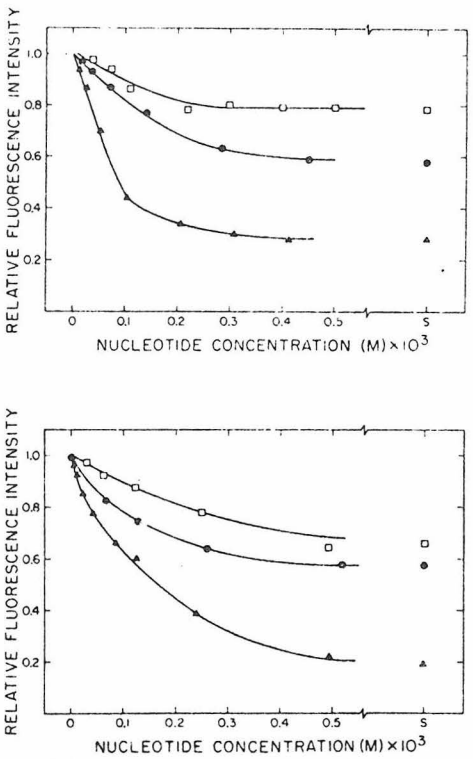

FIGURE 1: Relative fluorescence intensity of quinacrine measured in the presence of increasing concentrations of unfractionated chromatin (●), S2 euchromatin (▲), and P1 heterochromatin (□). The data for P2 heterochromatin (not shown) were essentially identical with that for unfractionated chromatin. Chromatin fractions were isolated after 15-min exposure to DNase. Quinacrine fluorescence was measured in 0.1 M phosphate buffer (pH 6.8) as described in Methods. Data are included for (a) rat liver (top panel) and (b) *Drosophila melanogaster* chromatin (bottom panel). "S" indicates computer fit value of fluorescence at infinite nucleotide concentration.

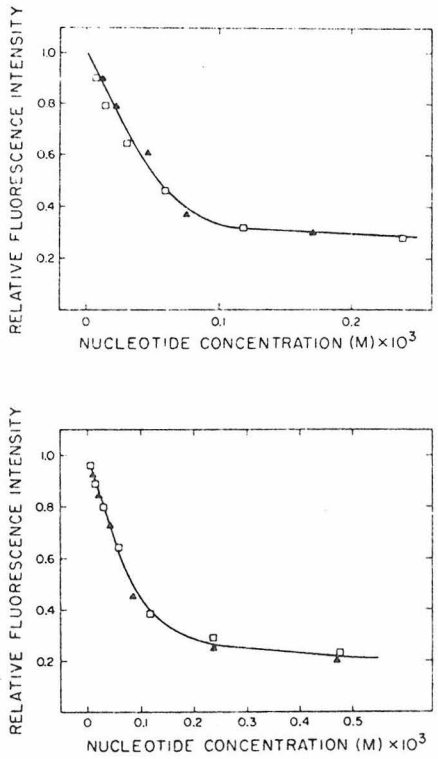

FIGURE 2: Relative fluorescence intensity of quinacrine measured in the presence of increasing concentrations of DNA prepared from S2 euchromatin (▲) and P1 heterochromatin (□). DNA was prepared from chromatin fractions obtained after 15-min exposure to DNase as described in Methods. Fluorescence was measured at a quinacrine concentration of 2×10^{-6} M in 0.1 M phosphate buffer (pH 6.8) as described in Methods. Data are included for (a) rat liver (top panel) and (b) *D. melanogaster* chromatin (bottom panel). "S" indicates computer fit value of fluorescence at infinite nucleotide concentration.

Pachmann and Rigler (1972) have presented absorption and emission spectra of quinacrine alone and quinacrine in the presence of both (A + T)- and (G + C)-rich polynucleotides.

QUINACRINE FLUORESCENCE IN THE PRESENCE OF CHROMATIN AND CHROMATIN FRACTIONS. Figure 1 presents the data obtained from fluorescence titrations of a standard amount of quinacrine (2.0×10^{-6} M) with varying amounts of chromatin and fractionated euchromatin and heterochromatin. Data are presented for two experiments, one performed with rat liver chromatin and another with chromatin prepared from 6- to 16-hr embryos of *Drosophila melanogaster*. The two chromatin preparations gave the same qualitative results. Euchromatin fractions from both rat liver and *Drosophila* embryo chromatin quenched quinacrine fluorescence to a greater extent than did either unfractionated chromatin or heterochromatin. The curves appear to be simple first-order saturation curves. We have performed a fit of the data by the least-squares method (straight-line fit of an inverse plot) to obtain fluorescence values at saturating concentrations of chromatin. The saturation value is the relative fluorescence of completely bound quinacrine. These results are also shown in Figure 1. The relative fluorescence of quinacrine bound to euchromatin is 0.19 for *Drosophila* and 0.28 for rat liver. Thus, the fluorescence of quinacrine is quenched by 70–80% on binding to euchromatin; on the other hand, quinacrine fluorescence is quenched by only 22–35% on binding to heterochromatin. Quenching by the nucleoprotein samples did not shift the emission maximum of quinacrine by more than 5 nm.

PROTEIN-DNA INTERACTIONS AND QUINACRINE FLUORESCENCE. We now attempt to answer the following question: Are the fluorescence patterns observed with quinacrine-nucleoprotein complexes due to differences in the base composition of the DNA of the chromatin fractions, or are they due in part to differences in protein-DNA interactions? The first half of this question can be answered directly by determining the base composition of the DNA isolated from the chromatin fractions. Furthermore, the fluorescence of quinacrine can be measured in the presence of these DNA samples.

The base composition of a DNA preparation (of 400 base pairs or greater in length) can be determined by measuring its T_m (mean temperature of thermal denaturation) in standard saline-citrate (Mandel and Marmur, 1968). Rat DNA (42% G·C) theoretically should exhibit a T_m of 86°. We observed a T_m of 85° for rat liver DNA. The DNA prepared from rat liver euchromatin (5-min DNase treatment) exhibited a T_m of 84°. Similarly, the DNA of rat liver P1 heterochromatin had a T_m of 85°. To a first approximation, therefore, the base composition of the DNAs from the chromatin fractions are identical (with 42 ± 2% G·C).

When the fluorescence output of quinacrine was measured in the presence of varying amounts of euchromatin and heterochromatin DNA, the saturation curves of Figure 2 were obtained. Data for euchromatin and heterochromatin DNA of rat

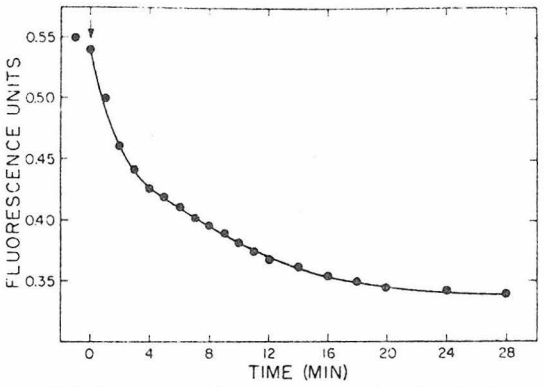

FIGURE 3: Time course of Pronase treatment of a quinacrine-heterochromatin mixture. At time zero (arrow), 50 μl of a 200-μg/ml solution of Pronase was added to 500 μl of a solution containing quinacrine (2×10^{-6} M) and rat liver P1 heterochromatin (83 μg/ml of nucleic acid). The heterochromatin sample was that obtained after 5-min exposure of chromatin to DNase. Other experimental details are given in Methods. Fluorescence is expressed in arbitrary units.

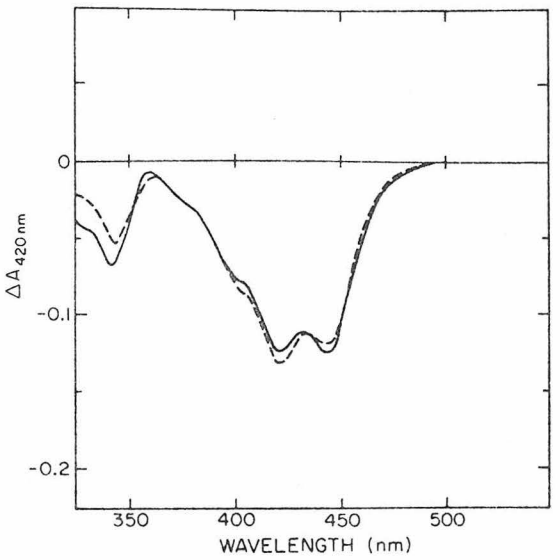

FIGURE 4: Visible difference spectra of quinacrine in the presence of near saturating concentrations of rat DNA (0.31×10^{-3} M in nucleotides) and chromatin (0.23×10^{-3} M in nucleotides) as compared to free quinacrine. Absorption spectra were measured at a quinacrine concentration of 4.4×10^{-5} M in 0.01 M Tris-Cl (pH 8). DNA = solid line; chromatin = dashed line.

(Figure 2a) and *Drosophila* (Figure 2b) are shown. As expected from base composition determinations, the data for euchromatin and heterochromatin DNA (from the same organism) are identical. The fluorescence titration curves for unfractionated DNA were also found to be the same as those for euchromatin and heterochromatin DNA from the same organism. The fluorescence titrations for euchromatin (Figure 1) most closely resemble those for deproteinized DNA (Figure 2), and the relative fluorescence of quinacrine at saturating concentrations of rat euchromatin (Figure 1a) was the same as that value at saturating concentration of rat DNA (Figure 2a).

Since base composition differences cannot reasonably account for the data of Figure 1, some other component of chromatin must be responsible. In order to examine the role of chromosomal proteins, the fluorescence of a quinacrine-heterochromatin mixture was monitored with time after addition of Pronase to the solution (Figure 3). Over a 20-min period a marked decrease in fluorescence output was noted. This is the change one would expect as heterochromatin is deproteinized and transformed into DNA. Pronase by itself did not alter the fluorescence of quinacrine. Furthermore, chromosomal proteins by themselves (histone and non-histone proteins) did not affect quinacrine fluorescence under our experimental conditions (0.01 M Tris-Cl (pH 8) or 0.1 M sodium phosphate (pH 6.8)). Thus it appears that protein-DNA interactions in chromatin are responsible for the observed results (Figure 1).

We have indicated that the fluorescence of quinacrine bound to heterochromatin is substantially greater than the fluorescence of quinacrine bound to euchromatin or DNA. We attribute this difference to the different quantum yields of the bound species. This conclusion rests on the value of relative fluorescence at saturating nucleotide concentrations. However, if the fractions are vastly different with respect to either binding affinities for quinacrine or the number of sites per nucleotide available for binding, the conclusions reached above must be reassessed. Therefore, a quantitative estimation of these binding parameters has been obtained.

Binding of Quinacrine to Chromatin and DNA. SPECTRAL CHANGES ON BINDING. Quinacrine in aqueous solution has absorption maxima at 345, 424, and 446 nm. On binding to DNA, the absorption maxima are shifted to longer wavelengths; moreover, the entire spectrum undergoes a hypochromic shift. This is characteristic of the interaction of planar dye molecules with nucleic acids (Blake and Peacocke, 1968). Pachmann and Rigler (1972) have presented data which indicate that both (G + C)- and (A + T)-rich polynucleotides have the same effect on the absorption spectrum of quinacrine as naturally occurring DNA (<60% A·T).

On binding to either chromatin or the chromatin fractions, the absorption spectrum of quinacrine undergoes the same hypochromic shift as on binding to rat DNA. Absorption difference spectra are presented in Figure 4. The absorption spectra of quinacrine-DNA and quinacrine-chromatin complexes were measured at near saturating nucleotide concentrations. Under these conditions nearly all the dye present in solution is bound. Spectra were taken with two cuvets in the reference position of the spectrophotometer; one cuvet contained quinacrine alone while the second cuvet contained either DNA or chromatin. An instrument baseline was obtained by measuring the absorption of quinacrine with an identical cuvet containing quinacrine in the reference position. The absorption difference spectra of quinacrine-DNA and quinacrine-chromatin complexes are nearly identical (Figure 4). Thus it appears that quinacrine forms a similar complex with chromatin as with DNA. Lerman (1963) has shown that the mode of interaction of quinacrine with DNA is intercalation. It is tempting, therefore, to speculate that the mode of interaction of quinacrine with chromatin is also *via* intercalation of the dye between the stacked bases of DNA. Since the absorption spectrum of quinacrine undergoes the same transition upon interaction with DNA or chromatin, spectrophotometric titrations can be used to investigate the binding of quinacrine to DNA and nucleoproteins quantitatively.

A spectrophotometric titration of quinacrine with increasing concentrations of rat chromatin, euchromatin, and heterochromatin was performed in order to obtain the value of the extinction coefficient of bound quinacrine. Figure 5 and Table II present the results of this experiment. The values of $\epsilon_{420\,nm}{}^{b}$ are nearly identical (±3.5%) for DNA, chromatin and the chromatin fractions. Even though the absorption values at sat-

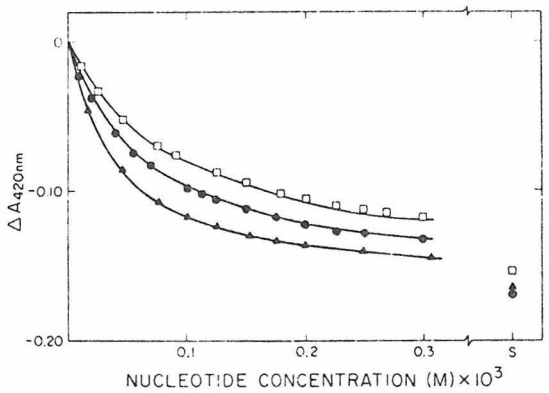

FIGURE 5: Spectrophotometric titration of quinacrine (4.4×10^{-5} M) with increasing concentrations of rat chromatin (●), P1 heterochromatin (□), and S2 euchromatin (▲). The absorption of quinacrine in 0.01 M Tris-Cl (pH 8) in the absence of nucleoproteins was 0.335 at 420 nm. "S" indicates computer fit value of absorption at infinite nucleotide concentration. Chromatin fractions were obtained after 15-min exposure to DNase.

uration are nearly identical for the nucleoprotein preparations, the spectrophotometric titrations are somewhat different. An equivalent hypochromic shift in the absorption of quinacrine was found at lower concentrations of euchromatin than heterochromatin; that is, more heterochromatin than euchromatin was required to produce the same decrease in absorption. This holds true for nonsaturating concentrations (less than 3×10^{-4} M in nucleotides). Again, quinacrine fully bound to chromatin, euchromatin or heterochromatin has essentially the same extinction at 420 nm.

SCATCHARD PLOTS. Spectrophotometric titrations were

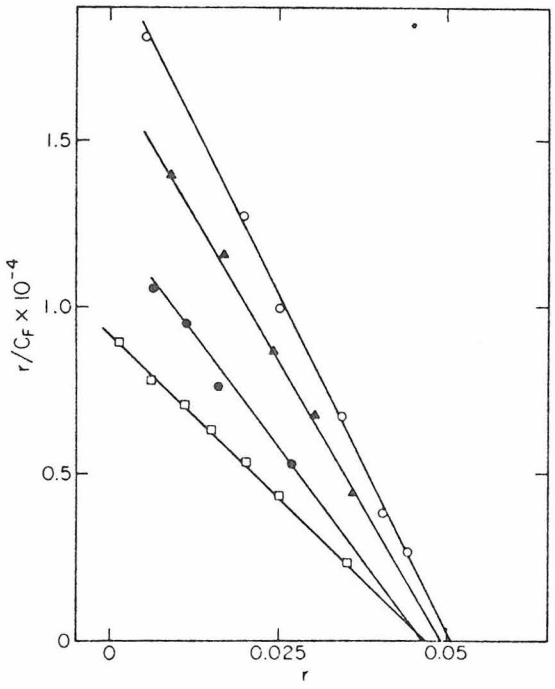

FIGURE 6: Scatchard plots of data obtained from spectrophotometric titrations of a standard amount of DNA or chromatin with increasing concentrations of quinacrine (see Methods): DNA (○); unfractionated chromatin (●); S2 euchromatin (▲); and P1 heterochromatin (□). Chromatin fractions were obtained after 15-min exposure to DNase.

TABLE III: Data from the Scatchard Plots.

Sample	K_a (10^5 M^{-1})	K_d (10^{-6} M)	$\bar{n}$ (Sites/ Nucleotide)	Sites/10 Base Pairs
DNA	4.0	2.5	0.051	1.02
Chromatin	2.6	3.9	0.047	0.94
Euchromatin	3.5	2.9	0.049	0.98
Heterochromatin	2.0	5.0	0.047	0.94

also performed under conditions where the concentration of nucleic acid was fixed and the concentration of quinacrine was varied over a 15-fold range (see Methods). The results of such titrations were plotted according to the method of Scatchard (1949). Figure 6 illustrates Scatchard plots for rat DNA, chromatin, euchromatin and heterochromatin. The intercept on the r axis (the abscissa) is $\bar{n}$, the maximal number of moles of quinacrine bound per mole of nucleotides, and the slope is $-K_a$, the association constant. The values of $\bar{n}$ obtained for DNA, chromatin and the chromatin fractions are nearly identical (Table III); the average value of $\bar{n}$ was 0.048 ± 0.002. This corresponds to about one site available for quinacrine binding per turn of double-helical "B"-form DNA.

The association constants obtained from the Scatchard plots are listed in Table III. The values of K_a for the interaction of quinacrine with DNA and for quinacrine with heterochromatin differ by only a factor of two. The values of K_a for euchromatin and unfractionated chromatin lie between the values for DNA and heterochromatin. The data of the Scatchard plots appear to lie on one straight line; this indicates that only one class of binding sites for quinacrine exists in either DNA or chromatin (Blake and Peacocke, 1968). This finding was quite surprising since A·T and G·C base pairs affect the fluorescence of quinacrine so differently. From the Scatchard plots we must conclude that quinacrine binds to A·T and G·C base pairs by the same thermodynamic process. Furthermore, since the maximal number of sites available for quinacrine binding ($\bar{n}$) is the same for DNA, chromatin and the chromatin fractions, chromosomal proteins do not occupy potential binding sites. Chromosomal proteins do, however, decrease the association constant for the interaction of quinacrine with the nucleic acid in chromatin. These data are quite similar to those obtained by Simpson (1970) for the interaction of a reporter molecule with DNA and chromatin. The number of sites available for binding the reporter ($\bar{n}$) was found to be the same for DNA and chromatin; however, the association constant for the interaction of the reporter with chromatin was one-half that for the interaction of the reporter with DNA.

Pachmann and Rigler (1972) and Modest and Sengupta (1973) have also presented results of binding experiments with quinacrine and DNA. Using fluorescence polarization, Pachmann and Rigler (1972) report an association constant of 6-10 $\times 10^5$ M^{-1} and n = 0.08-0.16. These values are in close agreement with those of Figure 6 and Table III. On the other hand, Modest and Sengupta (1973) have reported values of $K = 1.6 \times 10^7$ M^{-1} and $n = 0.72$. These data were obtained by ultrafiltration and probably reflect the ionic interaction of quinacrine with DNA phosphates. The spectroscopic binding data reported herein and by Pachmann and Rigler (1972) reflect binding *via* intercalation. The second (ionic) class of binding sites was not detected in our experiments (Figure 6); higher dye to nucleotide ratios, however, have revealed this class of binding.

ESTIMATION OF BINDING PARAMETERS FROM FLUORESCENCE DATA. We have mentioned before that the binding parameters K_a and $\bar{n}$ cannot be determined independently from fluorescence titrations with natural DNAs due to the fact that A·T and G·C base pairs have different effects on quinacrine fluorescence. Hélène *et al.* (1971), however, have shown that the product of $K_a\bar{n}$ can be obtained directlyly from a titration of a fluorescent molecule with DNA. Equation 6 of Hélène *et al.* (1971) can be modified to give a general equation for the binding of fluorescent molecules to DNA

$$\phi_F/(\phi_F - \phi) = [\sum_i K_{a^i}\bar{n}_i\phi_F/\sum_i K_{a^i}\bar{n}_i(\phi_F - \phi_B{}^i)] + (1/C_{DNA})[\phi_F/\sum_i K_{a^i}\bar{n}_i(\phi_F - \phi_B{}^i)] \quad (1)$$

This equation assumes that i classes of binding sites exist. ϕ_F is the quantum yield of free quinacrine; $\phi_B{}^i$ is the quantum yield of quinacrine bound to sites of class i; and ϕ is the observed quantum yield. $\phi_F/(\phi_F - \phi)$ is equal to $1/\Delta f$, where Δf is 1 minus the quantum yield ϕ/ϕ_F. Equation 1 describes a straight-line plot of $1/\Delta f$ *vs.* $1/C_{DNA}$. Figure 7 illustrates such a plot for rat DNA and for poly(rG)·poly(rC). The ratio of y intercept to slope is $\Sigma_i K_{a^i}\bar{n}_i$. From Figure 7, $\Sigma_i K_{a^i}\bar{n}_i$ is 2.07×10^4 M^{-1} for rat DNA, and 2.21×10^4 M^{-1} for poly(rG)·poly(rC). From the Scatchard plot for rat DNA, K_a is 4.0×10^5 M^{-1} and $\bar{n}$ is 0.051. The product $(K_a\bar{n})$ is 2.04×10^4 M^{-1}. Thus by two independent measurements (fluorescence and spectrophotometric titrations), we arrive at the same value of $K_a\bar{n}$. This indicates that the values of K_a and $\bar{n}$ obtained from the Scatchard plots are reliable.

We must reemphasize that fluorescence titrations cannot be used to estimate K_a and $\bar{n}$ independently for *natural* DNAs. Binding parameters can be estimated, however, from fluorescence titrations of quinacrine with synthetic homopolymer duplexes. From a Scatchard plot of the fluorescence titration data for poly(dA)·poly(dT) (Weisblum and de Haseth, 1972), a K_a of 3.3×10^5 M^{-1} was calculated. This value is quite similar to the value of K_a calculated from the spectrophotometric titration for rat DNA (4.0×10^5 M^{-1}). Furthermore, the value of K_a for poly(rG)·poly(rC) is approximately 4.4×10^5 M^{-1}. Thus quinacrine appears to have about the same affinity for A·T and G·C base pairs. Moreover, the number of sites per nucleotide available for binding ($\bar{n}$) appear to be the same for all polynucleotides and nucleoproteins investigated ($\bar{n} = 0.048 \pm 0.002$).

DNA Conformation. CIRCULAR DICHROISM SPECTROSCOPY. In recent years circular dichroism spectroscopy has come into wide use as a tool for investigating nucleic acid conformation and protein-induced alterations in DNA conformation. We have measured the CD spectra of chromatin, euchromatin, heterochromatin, and DNA under a variety of model conditions. Figure 8 illustrates the CD spectra for these nucleoproteins and DNA isolated from rat liver. The CD spectrum of DNA in aqueous solution is characterized by a major positive band at 275 nm and a major negative band at 245 nm with a crossover at the wavelength of maximal absorption, 258 nm. A second positive band is centered at about 220 nm. The CD spectrum of chromatin differs from that of DNA in several respects. The absolute magnitude of the positive band at 275 nm is reduced from that of DNA in chromatin. By treating chromatin with deproteinizing agents (sodium dodecyl sulfate, NaCl, or proteolytic enzymes), one can transform the CD spectrum from that of chromatin to one resembling the CD spectrum of DNA (Shih and Fasman, 1970; Simpson, 1972; Henson and Walker, 1970). Below 250 nm, the major features of the CD spectrum of chromatin are the protein CD band at 220 nm and the nucleic acid shoulder at 245-250 nm.

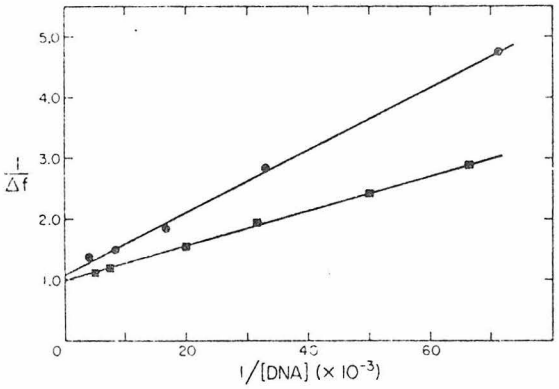

FIGURE 7: Plot of $1/\Delta f$ *vs.* 1/DNA concentration for rat DNA (●) and poly(rG)·poly(rC) (▣). The data for poly(rG)·poly(rC) were obtained from Weisblum and de Haseth (1972). $1/\Delta f$ is defined as the inverse of (1 − relative fluorescence intensity). The value of $\Sigma_i K_{a^i}\bar{n}_i$ was 2.21×10^4 M^{-1} for poly(rG)·poly(rC) and 2.07×10^4 M^{-1} for rat DNA.

The reduced value of the CD band at 275 nm has been interpreted by many authors as evidence for the altered conformation of DNA in chromatin. It has been suggested that the DNA in chromatin is partially in the "C" conformation (Johnson *et al.*, 1972). DNA is thought to exist in the "C" conformation under a variety of conditions; for example, Li salts of DNA at 95% relative humidity (Marvin *et al.*, 1961; Tunis-Schneider and Maestre, 1970) and DNA in ethylene glycol

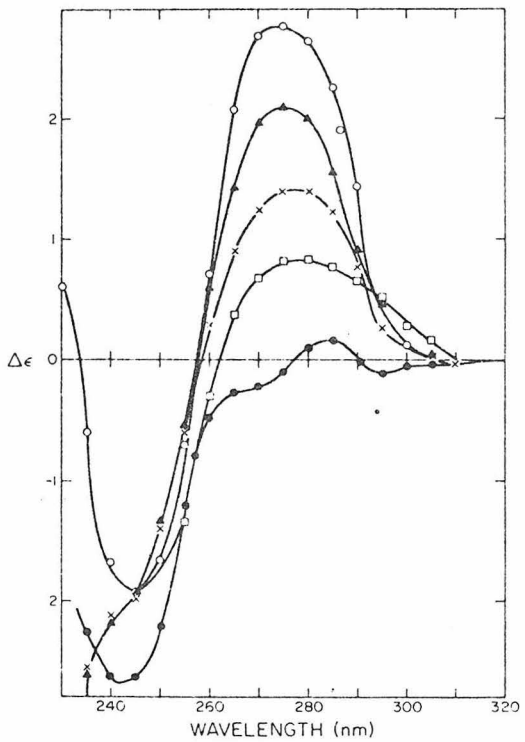

FIGURE 8: Circular dichroism spectra of rat DNA (○), chromatin (X), S2 euchromatin (▲), P1 heterochromatin (□) in 0.01 M Tris-Cl (pH 8) and rat DNA in 90% ethylene glycol (●). The chromatin fractions were obtained after 15-min exposure to DNase.

TABLE IV: CD and Nucleic Acid Conformation.

Sample	$\Delta\epsilon_{275\ nm}$	% B conformation
DNA, aqueous solution	2.64	100
DNA, 90% ethylene glycol	−0.01	0 ("C" form)
Chromatin	1.40	53
Euchromatin	2.00	76
Heterochromatin	0.83	32

(Nelson and Johnson, 1970) show characteristics of the "C" form. The CD spectrum of DNA in ethylene glycol (Figure 8 and Nelson and Johnson, 1970) is nearly identical the spectrum of films of Li salt DNA known to be in the "C" form (Tunis-Schneider and Maestre, 1970). We will therefore refer to the CD spectrum of DNA in ethylene glycol as a "C"-form spectrum. The B → C transition is characterized by an almost complete loss of the 275-nm positive band. Minor CD bands are seen at 284 and 295 nm, respectively. A negative shoulder is seen at 265–270 nm. The major negative band of DNA is increased in the "C"-form spectrum; the value of $\Delta\epsilon_{245\ nm}$ for "B"-form DNA is −1.96, while $\Delta\epsilon_{245\ nm}$ in the "C"-form spectrum is −2.67.

The values of $\Delta\epsilon_{275\ nm}$ for DNA ("B"- and "C"-form spectra) and the nucleoprotein samples are listed in Table IV. The spectrum of euchromatin is most like that of "B"-form DNA while heterochromatin exhibits a spectrum more like that of the "C"-form of DNA. The spectrum of unfractionated chromatin is intermediate between those of euchromatin and heterochromatin. Johnson *et al.* (1972) have assigned a per cent "B" contribution to chromatin DNA from the CD spectrum. If we assume that all the base pairs of rat DNA in aqueous solution are in the "B" form, and that all the base pairs of rat DNA in 90% ethylene glycol are in the "C" form, then we can ascribe a per cent "B"-form conformation to the nucleic acid of the nucleoprotein samples. Since protein does not contribute to the CD spectrum of DNA above 250nm, we make use of $\Delta\epsilon_{275\ nm}$ in making the assignment of conformation. Our only reservation in making these assignments is that we do not know what effects, if any, light scattering may have on the circular dichroism spectrum of chromatin (Dorman and Maestre, 1973). The heterochromatin fraction (P1) showed considerably more light scattering than either euchromatin or unfractionated chromatin ($A_{320}/A_{260} \geq 0.1$ for heterochromatin; $A_{320}/A_{260} < 0.02$ for euchromatin and unfractionated chromatin). It is clear that the CD spectrum of heterochromatin does not fit either the "B"- or "C"-form spectra above 285 nm; this tailing of the positive CD band to longer wavelengths is most likely due to light scattering (Dorman and Maestre, 1973). Thus, we do not know to what degree our assignment of per cent "B" conformation to heterochromatin DNA is influenced by scattering artefacts; nevertheless, the conformations of DNA in the chromatin fractions are clearly different (Polacow and Simpson, 1973).

Conclusions

In this study, we have concerned ourselves with the biophysical basis of differential staining of chromosomes with quinacrine. We have chosen to work with fractionated interphase heterochromatin and euchromatin. We have shown that the quantum yield of quinacrine bound to either unfractionated chromatin or heterochromatin is significantly greater than the quantum yield of quinacrine bound to either euchromatin or DNA in aqueous solution. Deproteinization abolishes this differential fluorescence; furthermore, no differences in DNA base composition were found between the euchromatin and heterochromatin fractions. These results cast doubt on whether the banding patterns seen with quinacrine-stained chromosomes are solely a reflection of intrachromosomal differences in DNA base composition. It must be stressed, however, that this work *does not* dispute the findings of Weisblum and de Haseth (1972) and Pachmann and Rigler (1972) pertaining to the *in vitro* base specificity of quinacrine fluorescence with purified nucleic acids. In fact, the very sharp (and very narrow) fluorescent bands seen on the Y chromosome of *Drosophila* (Vosa, 1970) could indicate the localization of (A + T)-rich satellite DNA.

Quinacrine bands are generally thought to be localized in the heterochromatic regions of chromosomes (Vosa, 1970; Adkisson *et al.*, 1971; Gagné *et al.*, 1971). Our results suggest that the conformation of the DNA in the heterochromatic portion of chromosomes is altered from that of the "B" to the "C" form. Chromosomal proteins, in particular the histones, are thought to induce and maintain this change in nucleic acid conformation (Simpson, 1972; Shih and Fasman, 1972; Henson and Walker, 1970; Simpson and Sober, 1970). Furthermore, our results suggest that quinacrine bound to heterochromatin is highly fluorescent while quinacrine bound to euchromatin is only weakly fluorescent. The alternation of heterochromatic and euchromatic regions along the chromatids of metaphase chromosomes would, therefore, lead to fluorescence banding patterns. Caspersson *et al.* (1972) have shown that the mitotic chromosomes of several cell types of the same organism have the same banding patterns. Thus, banding does not reflect the genetic activity of cells *per se*. More likely, banding reflects the ordered packaging of DNA into the chromosome.

One of the most remarkable features of chromosome banding techniques is the similarity of bands produced by a variety of methods. Lee *et al.* (1973) have identified four groups of techniques which produce similar patterns; these are (1) quinacrine fluorescence; (2) Giemsa in combination with alkali-heating techniques; (3) Giemsa in combination with any one of several proteolytic enzymes; and (4) Giemsa in combination with protein-denaturing substances such as 5 M urea and anionic and nonionic detergents. Until recently, it was thought that the Giemsa–alkali heating technique (number 2 above) reflected the differential chromosomal localization of classes of repetitious and nonrepetitious DNA. Comings *et al.* (1973) and Stockert and Lisanti (1972) have shown that *in situ* renaturation of chromosomal DNA (repetitive and single copy) is complete within a few minutes. Thus, preferential reassociation of repetitious DNA cannot explain the banding patterns produced with Giesma. Comings *et al.* (1973) have also shown that the G-banding techniques remove very little DNA or protein from the chromosomes. Harsh treatment of chromosomes with alkali or prolonged exposure to proteolytic enzymes abolishes G bands altogether.

The results of G-banding techniques employing mild treatment with proteolytic enzymes and detergents tend to support the idea that chromosome banding patterns do not reflect intrachromosomal differences in DNA base ratio. These agents (enzymes and denaturants) presumably act only on the protein component of chromosomes. The fact that these techniques give rise to banding patterns which are similar to those obtained with quinacrine support the conclusion that variations in protein DNA interactions along the chromatids of metaphase chromosomes are responsible for banding. Recently, Rodman and Tahiliani (1973) have shown that Feulgen staining of mouse chromosomes reveals a banding pattern similar to that

obtained with Giemsa or quinacrine-mustard. These authors postulate that "the localization of Feulgen dark and light stain, representing relative DNA densities, reflects the regional protein association of the DNA."

In conclusion, we suggest that chromosome banding with quinacrine reflects differences in protein-DNA interactions, and DNA conformation, along the chromatids of metaphase chromosomes.

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FURTHER DISCUSSION

Since this publication appeared, Comings et al. (1975) have presented results obtained from a similar study of the interaction of quinacrine with polynucleotides and chromatin fractions. In agreement with our results, Comings et al. observed that chromatin fractions, isolated by differential centrifugation of sonicated chromatin, differ in their ability to quench quinacrine fluorescence. DNA prepared from the various chromatin fractions quenched quinacrine fluorescence to the same extent. Equilibrium dialysis experiments suggested that the chromatin fractions differed in their ability to bind quinacrine; the poorest binding fractions were enriched in nonhistone proteins. In agreement with the observation that acid treatment (histone-extraction) does not alter Q-banding of metaphase chromosomes, histones had little effect on the quenching of quinacrine fluorescence by DNA. Agents that cause DNA to undergo the B $\rightarrow$C transition (salts, ethylene glycol) also prevent quinacrine binding and fluorescence quenching. Comings et al. have concluded that the most pale staining regions in metaphase chromosomes are due to a decreased binding of quinacrine and that this inhibition is predominantly due to nonhistone proteins. Our results are in general agreement with those of Comings et al.; however, we concluded that quinacrine banding was due to protein-DNA interactions and DNA conformation. Our data did not indicate which class of chromosomal proteins

might be responsible for quinacrine staining. In light of the results of Comings et al., it appears that the proteins responsible for banding are the nonhistones. Furthermore, the results of Comings et al. cast doubt on our hypothesis of the relationship between DNA conformation and quinacrine fluorescence. It seems more reasonable that poorly staining regions of chromosomes should bind less dye than the brightly staining regions. Nevertheless, our results and those of Comings et al. show that DNA-protein interactions are central to the mechanism of quinacrine staining of chromosomes.

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