DNA-binding non-histone proteins (NHP) from rat liver have been prepared by utilizing their affinity toward phosphocellulose. The specificity of the DNA binding sites was investigated by the reassociation kinetics of the protein-bound DNA. Isolated protein-bound DNA (350 nucleotides) reassociated with a 3-fold increase in the content of middle repetitive sequences as found in the whole rat genome. Kinetic analysis of the bound DNA reassociation suggests that half of the total repetitive sequences was concentrated by the NHP interactions. Saturation reassociation of isolated middle repetitive DNA or the whole rat genome, of various fragment lengths, with an excess of bound DNA indicates 5% or less of the rat genome is complementary with the driver DNA. This finding confirms the previous observations that a subset of the repetitive sequences is concentrated by NHP-DNA interactions (Sevall, J. S., Cockburn, A., Savage, M., and Bonner, J. (1975) Biochemistry 14, 782–789).

Increasing the DNA fragment length to 3400 nucleotides enhanced the amount of NHP-retained DNA. Reassociation of sheared bound 3400 nucleotide-DNA resulted in two kinetic components, the slower component being nonrepetitive and the fast component being repetitive. The repetitive sequences displayed reassociation kinetics identical with those of the NHP-bound 350-nucleotide fragments. The NHP-bound nonrepetitive component was 10% of the whole single copy sequences. Rat liver polysomal RNA-driven hybridization of the protein-bound nonrepetitive component had a 4- to 5-fold increase in complementary DNA fragments when compared with the whole rat genome nonrepetitive sequences even though this represents only 10% of the total genome.

The molecular structure of eukaryotic chromatin is characterized by DNA coiled around a histone core. These structures are referred to as nucleosomes. One difference between actively transcribed and nontranscribed nucleosomes lies in the susceptibility of the nucleosome to nuclease (DNase I or II) digestion (1-4). The structural and functional differences between active and inactive chromatin are presumed to depend upon differences in their content of DNA-associated proteins as evidenced by the fact that template-active chromatin digested with DNase I releases a subset of proteins which includes histones and a very specific subset of nonhistone proteins (NHP) (5). The template-active DNA contains a subset of sequences of the whole rat genome as shown by reassociation analysis (2, 4).

Native DNA-binding NHP associated with rat liver chromatin contain a very small subset of homologous DNA-binding NHP. This subset interacts preferentially with repetitive sequences of the rat genome (7). DNA-binding NHP have been implicated in both positive or negative roles in transcriptional regulation of specific genes (8, 9). The NHP may interact specifically with DNA or the nucleosome complexes thereby inducing a conformational change in chromatin (10, 11) or affecting the thermal stability of the DNA (12, 13).

An alternative approach to the elucidation of gene regulation is the determination of the sequence organization of the eukaryotic chromosome. Most animal genomes consist of interspersed repetitive and nonrepetitive sequences (14). The repetitive sequences contain a subset that may function as control element in gene expression (6). In the present study, rat DNA which is preferentially bound by a group of heterogeneous rat liver phosphobinding NHP is characterized by its reassociation kinetics and polysomal RNA hybridization.

**MATERIALS AND METHODS**

**Purification Techniques**—Non-histone proteins (NHP) were purified from chromatin isolated from rat liver nuclei (15).

The chromatin was dissociated in 4 M NaCl, 0.01 M Tris-HCl, pH 8.0. The DNA was removed by centrifugation, and the histones were removed by cation exchange columns (7). In all steps, diethylaminoethyl phosphate (0.1 mm) was added to inhibit protease activity (16). Phosphobinding NHP were isolated by phosphocellulose chromatography (Serva. N. Y.) with the retained proteins eluting between 0.2 and 0.6 M NaCl in a linear gradient. Polypeptide content was determined by denaturing gel electrophoresis (17).

Rat DNA was purified from crude chromatin by a modified Sevag procedure (18). The DNA was sheared to 350-nucleotide, single-stranded length in an Amincon French Press at 20,000 p.s.i. in 0.2 M sodium acetate, 66% glycerol at 0°C. Rat DNA was sheared to a single-stranded length of 3400 nucleotides by high speed blending at 5,000 rpm for 30 min in 0.1 M sodium acetate at 0°C in a VirTis 60 Homogenizer. DNA was sized by electron microscopy using a modified Kleinschmidt procedure (19), and weight average lengths were calculated by alkaline sucrose gradients according to the relationship derived by Studier (20). Five to twenty per cent linear sucrose gradients containing only 0.1 M NaOH were centrifuged in a SW 50.1 rotor at 40,000 rpm for 4.5 to 7 h depending on fragment size. Labeled DNA markers (Bethesda Research) were run in the same gradient as the sheared fragments. Weight average length values are reported in this paper.

Single copy rat DNA from the whole sheared rat genome was isolated by successive fractionation at Cst 200. In a whole rat DNA-driven reassociation, 93% of the tracer reassociated with a rate constant of 4.0 x 10^-6 mol -1 s^-1 which is identical with that of the single copy component of the whole genome.

**DNA Labeling**—A microscale procedure to synthesize and purify rat DNA to high specific activity was modified from the technique of Schachat and Hogness (21) and Mackey et al. (22). [³²P]DNA of specific activity 1.5 x 10⁹ cpm/μg was prepared by nick translation

*The abbreviations used are: NHP, non-histone protein; RNA, protein-bound DNA sequence.
using 0.1 nmol of commercial [3H]byridine 5'-triphosphate (50 Ci/ 
mol, ICN) and [3H]deoxythymidine 5'-triphosphate (20 Ci/mmol, ICN), 0.4 nmol of deoxyguanosine 5'-triphosphate and deoxyadenosine 5'-triphosphate (P-L Biochemicals), 66.7 mM potassium phosphate (pH 7.5), 6.7 mM magnesium chloride, 1 mg of unlabeled DNA, and 10 units of DNA polymerase I (Worthington Biochemicals or P-L Biochemicals). The final volume was 25 ml. After 6-h incubation at 14°C, 0.1 ml of 0.1 M EDTA was added to the mixture and the double- 
stranded DNA was precipitated with ethanol and hydroxylapatite column. The labeled DNA was eluted in 0.48 mM sodium phosphate buffer, pH 6.8. If the labeled DNA was to be used in reassociation studies, carrier DNA was added and the mixture was phenol-extracted, treated with Chelex, and prepared for reassociation.

The labeled DNA was characterized by its reassociation properties. The reassociation rate of labeled DNA, its complexity and component composition all agree with unlabeled DNA reassociation values.

Reassociation of Labeled with Unlabeled DNA and Assay of Duplex Formation—Samples were denatured at 100°C for 5 min and annealed in sodium phosphate buffer (PB) at 62°C for 0.12 mM PB, 72°C for 0.48 mM PB, or 94°C for 0.06 mM PB. In most experiments, labeled and unlabeled DNAs were reassociated. In such mixtures, the unlabeled DNA was present at greater than 10 times the concentration of labeled “tracer” DNA.

The reassociating DNA was incubated to different Ca concentrations (value (s) multiplied by DNA concentration (moles of nucleotides/liter)) calculated for the carrier DNA fragments. After incubation the samples were frozen in dry ice-ethanol for further analysis or immediately analyzed for duplex-content. The samples were diluted 2 to 5× with carrier DNA and passed over a hydroxylapatite column (Bio-
Rad, DNA grade) at 60°C. Single-stranded DNA was eluted in 10 ml of 0.19 mM PB. The duplexed DNA was eluted with 10 ml of 0.48 mM PB.

Five-milliliter filters were collected; 100 μg of unlabeled carrier RNA (Sigma Chemical Co.) was added to the mixture and the nucleic acid precipitated in ice cold 10% trichloroacetic acid. The precipitated nucleic acid was collected on 0.6-μm pore size Schleicher and Schuell membrane filters, washed with 1 ml of ethanol, dried in a vacuum oven, and counted in a xylene base scintillation cocktail. The percent single-stranded DNA was determined from total counts recovered.

The data for a single reassociation study were analyzed on a computer program capable of fitting several kinetic components with second or first order kinetics (24).

Protein-bound DNA Assay and Isolation—Protein-DNA complexes can be retained on nitrocellulose filters. The NHP and DNA (1 μg/ml) were mixed in a volume of 1.5 ml of assay buffer and allowed to equilibrate at 22°C for 15 min. Longer incubation times did not result in increased retention. Triplicate, 0.5-ml aliquots were filtered at 2 p.s.i. over Schleicher and Schuell B6 filters (24 mm diameter, 0.45 μm pore size) presoaked in the reaction buffer (1 mM phosphate buffer, 0.1% sodium dodecyl sulfate, and 100 mM sodium chloride). The proteins and DNA were mixed in reaction buffer at mass ratios reported in results. DNA concentration in the final mixture was 1 μg/ml. No more than 0.5 mg of protein was filtered over any one filter. The mixture was soaked at 37°C in 5 mM of 0.05% sodium dodecyl sulfate to remove BDNA. Less than 1% of the initial bound DNA was irreversibly bound to the filter. BDNA was concentrated over hydroxylapatite columns, lyophilized, and prepared for reassociation studies (25).

Messenger RNA Isolation and RNA-driven Hybridization Reactions—Polyinosinic messenger RNA was prepared from rat liver by a modification of the method of Lee and Brawner (26). Liver was homogenized in 5 ml 0.1 M Tris-HCl (pH 7.4), 0.1 M dithiothreitol, and 0.1 M sucrose, 0.25 M KCl, 0.01 M MgCl₂, 0.05 M Tris-HCl (pH 7.4), and 0.1% diethanolpyrocatechol. The nuclei and cell debris were removed by a 3000 x g, 10 min 0°C centrifugation; mitochondria were removed at 17,000 x g, for 10 min at 0°C. The polysomes were pelleted by centrifugation at 90,000 rpm for 90 min in a Spinc 50.2 Ti rotor. Pellets were suspended in 10 ml of 0.1 M EDTA and 1 mM mercury-hcl (27).

After 15 min, the supernatant was layered over a 20% sucrose, 0.1 M EDTA pad, and centrifuged at 30,000 rpm for 1 h in a type 30 rotor to remove >80 S particles. The supernatant was phenol-
extracted and ethanol-precipitated (28). The precipitate was resus-
pended in 0.4 I ml phosphate buffer, 0.1% sodium dodecyl sulfate, and employed in mRNA driven hybridizations.

The mRNA was quantified by measuring the amount of the isolated mRNA that could hybridize with excess single copy rat DNA. Forty-seven per cent of the mRNA was hybridized in a whole rat genome-driven hybridization. The unhybridized RNA was isolated and rehybridized with an excess of the whole rat genome. The mRNA preparation consisted of 64% of the hybridizable RNA which was taken as the fractional amount of hybridizable RNA in the RNA preparation (44, 45).

RESULTS

Fractionation of NHP—Non-histone proteins from nuclei of quick-frozen male rat livers were fractionated by gradient phosphocellulose chromatography. Ten per cent of the total NHP was eluted in a single peak and termed phosphocellulose-binding NHP. Addition of a DNA-cellulose column in tandem behind the phosphocellulose did not retain any additional DNA-binding NHP from the non-phosphobinding proteins. Sodium dodecyl sulfate denaturing gel analysis indicated that a subset of the NHP is found in the phosphobinding fraction of molecular weight 2 to 7×10⁶ (Fig. 1).

Fig. 2 indicates the retention of BDNA complexes with 3400-nucleotide pair and 350-nucleotide pair rat DNA on a phosphocellulose membrane filter. At increased protein/DNA ratios, a greater amount of rat DNA can be retained on the membrane filter. Since specific DNA-binding proteins have a low affinity nonspecific binding constant and a high affinity specific binding constant (33, 34), lower protein/DNA ratios would favor the formation of high affinity binding complexes. In low protein/DNA ratio range (1 to 5:1), the retention of DNA on the membrane filter approaches linearity with the slope of retention for the 3400-nucleotide fragment 5-fold greater than that for the 350-nucleotide DNA fragment. Linearity implies at least a one to one relationship of protein binding site/DNA fragment and the increase in the slope of retention due to DNA fragment size approaches the difference in the size relationship of the two DNA fragments. The NHP-bound DNA can be released from the membrane filter allowing isolation of the bound DNA for subsequent analyses (29).

Frequency Components of Bound DNA—Enrichment of a class of DNA sequences can be assayed by measurement of the rate of reassociation of the DNA sequences in question. For the following DNA reassociations, DNA duplexes were reassociated at 62°C, 0.18 M sodium ion criterion (33). As a reference, the reassociation of the whole rat genome, 350-nucleotide fragment length is shown in Fig. 3.

The total rat genome (sheared to 350 nucleotides) has three major components as reported previously (4, 36): a slow single copy region (A = 3.56 × 10⁻⁴ mol/s/liter⁻¹), a moderately repetitive region (second order kinetic components) and a very rapidly reassociating component. Since hydroxylapatite...
with respect to the entire rat genome. The repetition frequency of the repetitive component driven by the rat genome is $50,000$ (krp/ksc $= [20/3.6 \times 10^{-7}] = 50,000$) when compared to the single copy component. The remaining 20% of the 350-nucleotide-bound fragment reassociates with the nonrepetitive component of the whole rat genome.

**Bound DNA-driven Reassociation**—Isolation of a subset of repetitive sequences by the NHP interactions is confirmed by cross-reassociation of the isolated whole rat repetitive component with excess BDNA. The repetitive component of the rat genome was isolated as that component reassociating between the $C_{ot}$ values of 0.01 to 5. Self-reassociation of the isolated repetitive component of the rat genome is shown in Fig. 4A. Compared with internal standard of *Escherichia coli* DNA reassociation, 60% of the BDNA reassociated with complex kinetics (across 3 orders of magnitude of $C_{ot}$). Fifty percent of the reassociating DNA had a second order component of $1.4 \times 10^{-2}$ (moles/liter)$^{-1}$. The remaining 14% of the reassociating DNA can be fit to an additional second order component. The rate of the major component is 34 times faster than the whole rat genome “fast repetitive” component $[34 = k$ (pure component) $1.4 \times 10^{-2}/k$ (major repetitive component)] implying that the sequences were derived from a subset of the repetitive sequences of the whole rat genome.

Reassociation of trace BDNA with excess whole rat genome confirms an enrichment of repetitive sequences (Fig. 4B). Sixty percent of the tracer reassociates in the region of the middle repetitive component of the whole genome indicating that the content of repetitive sequences is concentrated 3-fold scores the entire DNA molecule as duplexed DNA, our components show increased rate constants when compared with S1 (single-stranded)-nuclease assays (36, 37). The curve through the data points is the best computer fit to a known single copy rate value of the slow component. The best fit analysis as measured by the lowest root mean square (rms) was 1.2% for the constrained fit. Two kinetic components can be resolved in the repetitive sequences, with the major component (fast repetitive component) of $k = 4.18$ (mol-s/liter)$^{-1}$. Information concerning the “fast repetitive component” is discussed by Pearson et al. (36) and Wu et al. (18). The majority of rapidly reannealing sequences have been thought to be foldback sequences in the rat genome (38).

Conditions for DNA isolation were set at $1 \mu$g of NHP/ug of 350-nucleotide DNA/ml where 1.7% of the input DNA was isolated as BDNA. The size of the DNA fragment was not altered after isolation as determined by alkaline sucrose gradients. Self-reassociation of the bound DNA is shown in Fig. 4A. Compared with internal standard of *Escherichia coli* DNA reassociation, 60% of the BDNA reassociated with complex kinetics (across 3 orders of magnitude of $C_{ot}$). Fifty percent of the reassociating DNA had a second order component of $1.4 \times 10^{-2}$ (mol-s/liter)$^{-1}$. The remaining 14% of the reassociating DNA can be fit to an additional second order component. The rate of the major component is 34 times faster than the whole rat genome “fast repetitive” component $[34 = k$ (pure component) $1.4 \times 10^{-2}/k$ (major repetitive component)] implying that the sequences were derived from a subset of the repetitive sequences of the whole rat genome.

Reassociation of trace BDNA with excess whole rat genome confirms an enrichment of repetitive sequences (Fig. 4B). Sixty percent of the tracer reassociates in the region of the middle repetitive component of the whole genome indicating that the content of repetitive sequences is concentrated 3-fold.

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**FIG. 1.** Sodium dodecyl sulfate polyacrylamide gels of non-histone proteins. 1, total nuclear sap proteins; 2, total non-histone proteins; 3, nonbinding non-histone proteins; 4, phosphobinding non-histone proteins. Molecular weight markers (Gallard-Schlesinger Chemicals) were run simultaneously with non-histone proteins.

**FIG. 2.** Protein-binding characteristics of rat DNA. $^3$H-labeled rat DNA (○, 3400 nucleotides; □, 350 nucleotides) at $1 \mu$g/ml was titrated with increasing amounts of non-histone proteins. Samples were incubated at room temperature (22°C) for 15 min in 0.15 M NaCl, 0.04 M PB, 0.15 M BMSH, $10^{-3}$ M EDTA, $10^{-4}$ M MgCl$_2$, pH 6.8. Triplicate 0.5-ml samples were filtered over $0.45\mu$m pore size membrane filters, washed, and counted. Data are reported as the average of triplicate samples.
Non-histone Protein DNA Interactions

Fig. 4. Reassociation kinetics of 350-nucleotide NHP-bound DNA. Data were obtained at various DNA concentrations in 0.12 M PB at 62°C. The protein-bound DNA was nick-translated (●). Reassociation of E. coli DNA (○) as an internal standard was monitored by optical density (35). The Cot half of E. coli DNA was 4.2 m s. B. reassociation kinetics of [3HINHP-bound DNA in an excess of unlabeled rat DNA. [3H]-labeled bound DNA was mixed with a 4000-fold excess of 350 nucleotide-long whole rat DNA (35). The data were obtained at various DNA and phosphate concentrations and were normalized to Cot values for 0.12 M PB at 62°C. Reassociated DNA samples were fractionated over hydroxylapatite. The abscissa refers to Cot values of driver DNA (Fig. 3).

Fig. 5A. Greater than 80% of the repetitive DNA can reassociate with kinetics expected of the pure repetitive component (k = 19 (mol s/liter)⁻¹). These data agree with published kinetics of the repetitive component in the rat genome (18, 36). The NHP-bound DNA (350 nucleotides) drove the reassociation of a trace amount of the [3H]-labeled whole repetitive component (Fig. 5B). Tracer reassociation was subtracted from the observed rate. Twenty-six per cent of the whole repetitive component reassociated with the major kinetic rate component of the DNA driver. The unassociated repetitive DNA could be reassinged in a whole genome excess of the hybridization when reassinged (data not shown). Thus, the repetitive component of the bound DNA reassociates with approximately 5% of the total rat genome [21% (total rat genome) × 26% (reassociated) = 5.2%].

Repetic sequences in the rat genome are interspersed among the nonrepetitive sequences (36). With the BDNA as driver, labeled rat DNA at various lengths was reassinged to a Cot of 0.50 (Fig. 6). The y axis is a measure of the amount of DNA at specific fragment length reassinged whereas the x axis indicates the fragment length of the labeled trace DNA (14). The intercept on the vertical axis of the line going through the points for fragments of 250 to 1000 nucleotide pairs represents the minimum fraction of NHP-bound repetitive sequences that is driving the reassigion (~2.0% of the rat genome). This value is consistent with the amount of whole rat repetitive component that reassociates with an excess of the BDNA fragment (5% of the whole genome). At a fragment length 2000 to 12,000 nucleotide pairs, the slope is decreased with an inflection point in the retention of the

Fig. 5. Reassociation kinetics of repetitive DNA. A, reassociation of repetitive DNA. Repetitive DNA was isolated by incubation of whole rat DNA to Cot 100 followed by fractionation over hydroxylapatite. Rapidly reassociating DNA was stripped from the repetitive DNA sample. Repetitive DNA was [3H]-labeled as described under "Materials and Methods" (21). B, reassociation kinetics of repetitive DNA in the presence of an excess of protein-bound DNA (350 nucleotides). [3H]-labeled repetitive DNA was mixed with a 900-fold excess of 350 nucleotide protein-bound DNA. Data were obtained in 0.12 M PB at 62°C.

Fig. 6. Interspersion analysis of 350-nucleotide NHP-bound DNA. [3H]-DNA fragments of varying size isolated by Agarose A-50 and alkaline sucrose gradient fractionation (35, 41) were reassigned in the presence of 100- to 1000-fold excess of NHP-bound DNA to a Cot of 0.5 m s. At this Cot all repetitive DNA sequences are reassigned. Reassociated DNA samples were fractionated by hydroxylapatite. Linear regression analysis was employed on the reassigion data of fragments 100 to 1000 nucleotides long and fragments 800 nucleotides or longer. The plotted data and the two linear regression lines are shown.
tracer DNA at approximately 1500 nucleotides. This inflection point indicates the bound repetitive sequences are interspersed among nonrepetitive DNA of 1500 nucleotides. The intercept on the y axis of the line going through the data for fragment lengths of 2,000 to 12,000 nucleotide pairs (approximately 19%) represents the amount of DNA organized in this interspersion pattern. The rising slope of retention with the longer fragment lengths implies longer interspersion of the bound sequences occurs in the rat genome. Scatter of the data points with the longer lengths prevents further analysis of this data.

**Long Bound DNA Fragments**—Interspersion of the bound repetitive sequences with single copy DNA imply that increased BDNA fragment length would increase the probability of including single copy sequences in the DNA fragment. Increasing the DNA fragment to 3400 nucleotides increases the amount of DNA retained by the NHP (Fig. 2). Under protein/DNA ratios that retained 1.7% of input 350-nucleotide DNA, 5% of the input 3400-nucleotide DNA was retained as protein-DNA complexes. Fig. 7A shows the reassociation of the unshredded isolated 3400-nucleotide DNA. Forty per cent of the DNA contained regions that could reassociate according to complex second order kinetics. The single-stranded DNA at Cot of 1000 was isolated and could reassociate with an excess of the entire rat genome to >95%. Sequences in high abundance reassociate under the set conditions while those in low abundance fail to reassociate (39). Those sequences in high abundance are apparently selected by the protein-DNA interactions.

**Fig. 7.** Reassociation kinetics of 3400-nucleotide NHP-bound DNA. A, reassociation of 3400-nucleotide bound DNA. Protein-bound DNA 3400 nucleotides long was self-reassociated and fractionated over hydroxylapatite. B, reassociation kinetics of sheared 3400-nucleotide NHP-bound DNA. The 3400-nucleotide NHP-bound DNA was sheared to 350 nucleotides as under “Materials and Methods.”

**Fig. 8.** Reassociation of fractionated 3400-nucleotide protein-bound DNA in whole genome excess. The fast (○) and slow (○) components of the sheared protein-bound DNA were reassociated in the presence of 10^5 mass excess of the whole rat genome. The dashed line is the reassociation of the whole rat genome as determined by optical density. The components were isolated as described in the text.

**Fig. 9.** Hybridization of [3H]DNA by polysomal messenger RNA. Data were fit to \( \frac{D}{D_0} = e^{kC_{ot}} \) where \( D \) is the concentration of unreassociated DNA and \( D_0 \) is the initial DNA concentration (24). Several preparations of mRNA were employed (○ and ●). A, the nonrepetitive component of the whole rat genome is the tracer DNA; B, the nonrepetitive component of the sheared protein-bound 3400-nucleotide rat DNA is the tracer. ● indicates different preparation of the protein-bound nonrepetitive sequences isolated by reassociation of sheared 3400-nucleotide protein-bound DNA to Cot = 1.0 m-s then incubating the single to a stranded DNA to Cot = 5000 m-s. Reassociation in excess whole genome is shown in Fig. 8.

Shearing the 3400-nucleotide BDNA to 350 nucleotides, and following its self-reassociation resolved two kinetic components (Fig. 7B). The slow component was 25% of the total BDNA with \( k = 3.4 \times 10^{-3} \) (mol·s/liter)^{-1} and the fast component in equal amount (25%) has a \( k = 90 \) (mol·s/liter)^{-1}. The BDNA had a different ratio of fast to slow reassociating sequences than the whole rat genome (1 part fast to 3 parts slow). Again, 70% of the sheared bound DNA reassociated by a Cot of 1000. The single-stranded DNA was again isolated and could be hybridized to both the repetitive (30%) and single copy component of the whole rat genome (70%).

The reassociating components were separated, the fast component by collecting the double-stranded duplexes at Cot = 1.0, and the slow component by collecting the single-stranded DNA at Cot = 1.0 followed by a second round of reassociation to a Cot = 1000. Fig. 8 shows the two isolated components when reassociated as tracer DNA with the whole rat genome. The fast component reassociates with the repetitive component. The slow component reassociates with a \( k = 4 \times 10^{-4} \) as
does the nonrepetitive component of the whole rat genome which represents an 8-fold \(3.4 \times 10^{-7}/4 \times 10^{-3}\) enrichment of single copy sequences.

**Functional Significance**—Transcribed sequences were assayed by polysomal RNA-driven hybridizations. As has been reported previously (Fig. 9A), 4.2% of the whole single copy DNA could hybridize with mRNA-driven hybridizations. Fig. 9B indicates 21.0% of the slow NHP-bound component is complementary to mRNA sequences. This mass ratio of polysomal RNA to DNA was greater than 10^{-2.4}, preventing any appreciable self-reassociation of the tracer. The RNA-DNA hybrids were assayed by hydroxyapatite which would score any duplexed DNA fragment as a hybrid; therefore, the actual amount of DNA complementary to polysomal RNA would be exaggerated (43). Direct comparison of the saturation plateaus indicates a 4- to 5-fold enrichment of the complementary sequences in the protein-bound slow component which is consistent with an 8-fold enrichment of single copy sequences by reassociation kinetics.

**DISCUSSION**

DNA binding non histone proteins have been shown to interact preferentially with the repetitive sequences in the rat genome (7). Some of the questions that should be posed concerning these sequences include: Is a subset of repetitive sequences isolated by the NHP-DNA interactions? Do the protein-DNA interactions have any functional significance?

DNA reassociation analysis of the isolated NHP-bound DNA can be used to answer the first question. Under stringent ionic strength conditions, 60% of the nucleotide protein-bound DNA reassociated with the kinetics of the repetitive driver in the whole rat genome. Thus, the BDNA is enriched in repetitive sequences. This finding confirms the repetitive preference of the DNA-binding NHP (7, 25). The repetition frequency of the major reassociating component is 50,000 as determined by the ratio of the repetitive rate to the single copy rate. Thus, the repetitive sequences bound by the NHP represent a highly repetitive group of sequences. Further, the increase in the observed rate in the self-driven reassociation of the protein-bound DNA is what is expected of an isolated kinetic component of the whole genome.

Protein-DNA interactions are characterized with low and high affinity constants. For DNA, the low affinity constants would be expected to be nonspecific. In the whole rat genome-driven reassociation, 20% of the bound sequences reassociate as nonrepetitive. The self-reassociation of the BDNA has 80% of the DNA reassociating as concentrated repetitive sequences whereas the kinetics do not detect any enriched single copy sequences. The single copy sequences detected in the driven reassociation must have a very high complexity and do not reassociate under the self-reassociation conditions. These sequences may be nonspecifically bound. We confirm earlier observations that rat liver NHP can interact with repetitive sequences in the rat genome (7).

To demonstrate a subclass of the repetitive sequences is isolated by NHP-DNA interactions it is, first, drive the entire genome or the repetitive sequences of the rat genome with an excess of the BDNA sequences. BDNA reassociates with 26% of trace \(^3\)H-labeled total repetitive component of the rat genome. One-fourth of the whole repetitive component was isolated by interaction with the NHP. This figure represents 5% of the rat genome isolated by protein-DNA interactions. Second, repetitive sequences in the rat genome are interspersed in the nonrepetitive sequences (18, 36); interspersion studies of the NHP binding sites show the bound repetitive sequences are also interspersed with nonrepetitive sequences.

Less than 3% of the NHP-bound repetitive sequences is interspersed approximately every 1500 nucleotides of nonrepetitive sequences across ~20% of the rat genome. Thus, the NHP-DNA interactions apparently concentrate a subset of repetitive sequence families.

Repetitive sequences in eukaryotic organisms are generally found in a specific sequence arrangement (40) and in distinct size classes (41, 42). The average arrangement in the rat genome has 70% of the rat genome with repetitive sequences interspersed among 2000- to 2700-nucleotide single copy DNA lengths (36). Isolating NHP-bound rat DNA fragments of 3400 nucleotides increases the probability of the DNA fragment containing a single copy element neighboring the repetitive binding site. Under the same protein to DNA concentrations as with 350-nucleotide DNA, 3400-nucleotide DNA was isolated as NHP-bound DNA. Self-reassociation of the 3400-nucleotide fragment showed 60% of the BDNA in duplex by a Cot of 1000. The remaining single-stranded DNA was reassociable but apparently was present in too low a concentration to reassociate. Shearing the 3400-nucleotide BDNA to 350 nucleotides resolved two kinetic components in equal proportions. Excess whole rat genome reassociation of the isolated components confirms the fact that the fast component is repetitive with similar kinetic parameters as the NHP-bound 350-nucleotide DNA and the slow component is comprised of nonrepetitive sequences with a complexity one-eighth that of the whole single copy component. Kinetic comparison entails a 2-fold standard error so enrichment ranges from 4- to 16-fold over the whole rat genome.

The rat genome contains two size classes of repetitive sequences (36): 40% of them are greater than 1500 nucleotides and 60% of them are 350 nucleotides in length. There is homology between the two size classes of repetitive sequences which is consistent with the concept that the short repetitive sequences are present within the long repetitive DNA sequences in the rat genome (18). The protein-bound 3400-nucleotide fragments have an equal amount of repetitive component as nonrepetitive component when sheared. This equal amount is unexpected if only 350-nucleotide repetitive sequences are bound since the DNA fragment is ~10-fold larger. However, isolation of a mixture of long and short repetitive sequences would increase the content of repetitive sequences in the long protein-bound DNA. This apparent inconsistency is now being investigated.

Is there any functional significance to the NHP-DNA interaction? Polysomal RNA-driven hybridization indicates a 4- to 5-fold increase of transcribed sequences is found in the NHP-bound slow component when compared with whole genome slow component. This is consistent with the observed increase in the BDNA single copy complexity. The use of the purified nonrepetitive component of the sheared 3400-nucleotide protein-bound DNA with a large (10^{5}) mass excess of polysomal RNA and measurement of DNA-DNA self-reassociation by RNaac A digestion rules out the possibility of a self reassociation artifact. The hydroxyapatite assay yields higher values for the amount of DNA complementary to RNA, but in comparison with the whole genome nonrepetitive component, an increase in the amount of transcribed sequences can be found in the nonrepetitive component of the 3400-nucleotide BDNA fragments.

The presence of positive regulators in NHP that affect the ovalbumin (46) and the histone (47) gene systems and negative regulators in the NHP fraction of Ehrlich ascites tumor chromatin (8, 9) has led to the investigation of those NHP that interact with high affinity toward homologous DNA. In this report, an assay for functional binding sites is reported for
positive NHP regulators. As improved protein fractionation techniques for NHP become available, the NHP binding sites can be assayed for contiguous functional genes.

Acknowledgments—The skillful technical assistance of Mr. Michael C. Carlsen is gratefully acknowledged. We are indebted also to Drs. W. R. Pearson and H. Popkin for computer analysis and to Drs. D. Stocco, J. Morrow, and J. R. Wu for careful reading of this manuscript.

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