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DNA sequence selection by tightly-bound nonhistone chromosomal proteins

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ABSTRACT

Extraction of chicken reticulocyte chromatin with 2.0 M NaCl removes 96% of chromosomal protein and yields two DNA components after dialysis and high-speed centrifugation. The bulk of chromosomal DNA (ca. 99%) is rendered free of protein, and is thus soluble in 10 mM Tris-HCl, pH 8.0. The other component (ca. 1%) displays a high protein/DNA ratio, and is insoluble in 10 mM Tris-HCl, pH 8.0. These DNAs can be separated on the basis of their solubilities. Analysis of the reassociation kinetics with total chicken DNA of these DNAs reveals marked differences. Whereas total DNA and the soluble component (DNA-S) have rapidly reassociating components, the insoluble component (DNA-P) is devoid of these components, and is therefore composed completely of unique sequence DNA. $C_0t_{\frac{1}{2}}$ values indicate that DNA-S is substantially depleted of some DNA-P sequences. We conclude that this segregation, as determined by tightly-bound nonhistone chromosomal proteins, selects a subset of total genomic DNA sequences, and suggests sequence-specific interaction between the tightly-bound nonhistones and DNA.

INTRODUCTION

Differential gene activity in eukaryotic cells has been thought to be at least in part the result of sequence-specific interactions between regulatory molecules and DNA. Of the protein components in chromatin, the histones are incapable of sequence recognition, and are thus unlikely candidates as regulatory molecules in this scheme (2). The suggestion that the nonhistone chromosomal proteins played a role in gene regulation originally emerged from chromatin reconstitution experiments (see review in (3)). As a result, much work has been done in recent years in the study of the nonhistones and their interactions in chromatin. This laboratory has been particularly interested in a specific subset of these proteins: the tightly-bound NHCP[1]. Tightly-bound in this report is an operational definition only, referring to solvent conditions required for extraction from chromatin, without regard for thermodynamic considerations.

Previous work from this laboratory (4,5) established that this group

of proteins is resistant to extraction from chromatin under conditions which extract the vast majority of chromosomal proteins, and this class of proteins is capable of DNA-binding activity in vitro without the requirement for the presence of histones, as compared to the absolute requirement for histones in the binding activity of most other NHCP. These tightly-bound NHCP (designated M_3 by this laboratory, since they can be extracted from chromatin in 5.0 M urea - 3.0 M NaCl) were found to directly stimulate transcriptional activity in vitro (6), and in chicken reticulocytes that DNA fraction associated with M_3 exhibited highly significant enrichment in globin gene sequences (7). Therefore, the combined data strongly suggest a regulatory role for M_3.

In this report, we have carried this work further by examining the nature of the DNA sequences retained by these proteins in chicken reticulocytes after extraction of the majority of the chromosomal proteins in 2.0 M NaCl. The interaction of these proteins with DNA is largely and strongly hydrophobic and thus they are resistant to extraction with this ionic solvent. We find that although only a small percentage of DNA remains protein-bound (ca. 1%), the tightly-bound NHCP select a defined subset of total unfractionated DNA sequences, in which only a portion of total DNA sequences are present. Whereas total unfractionated DNA and that fraction rendered virtually protein-free and thus solubilized display the presence of rapidly-reassociating sequences, these sequences are absent from protein-bound DNA (DNA-P). We also find that the soluble component (DNA-S) is substantially depleted of some DNA-P sequences, as indicated by comparison of the $C_0t_{\frac{1}{2}}$ values for the reassociation of DNA-P with total unfractionated DNA and DNA-S as drivers. Taken together, the data reported herein indicate that: (1) the tightly-bound NHCP are nonrandomly distributed in chromatin DNA, (2) the tightly bound NHCP are exclusively associated with unique-sequence DNA, and (3) the unique sequence DNA determined by the tightly-bound NHCP is an incomplete subset of total chicken unique-sequence DNA. This suggests a sequence-specific interaction between the M_3 proteins and DNA in the chicken genome.

MATERIALS AND METHODS

Preparation of Reticulocyte Chromatin: Adult chickens were rendered anemic by daily injection of phenylhydrazine (1 mg/kg) for a period of seven days. After this period, the chickens were decapitated and bled. Cells were

collected by centrifugation at 2,000 x g for 10 minutes. The cellular pellet was washed with ice-cold deionized water to lyse contaminating red blood cells. The cellular pellet was further washed with NET[1] and repelleted. The cells were lysed in RSB[1] containing 0.2% Triton X-100, and nuclei were obtained by centrifugation at 10,000 x g for 10 minutes. The nuclear pellet was washed three times with RSB without Triton. The final nuclear pellet was homogenized into TPD[1], nuclei being lysed with gentle homogenization. Crude chromatin was allowed to swell for 20 minutes, and was collected by centrifugation at 10,000 x g for 10 minutes. Nuclear lysis was confirmed by phase contrast microscopy. The crude chromatin pellet was taken up in TPD and layered on a cushion of 1.1 M sucrose-TPD, with centrifugation at 2,500 rpm for 15 minutes in the Beckman SW-27 rotor. Bulk chromatin remaining at the interphase was collected, and the nucleolar pellet was discarded. Bulk chromatin was layered on 1.7 M sucrose-TPD, centrifuged at 26,000 rpm in the SW-27 rotor for 90 minutes, and the final chromatin pellet was used for protein extraction. All operations were carried out at 0-4° C.

Extraction of Chromosomal Proteins: Purified chromatin from above was homogenized into 2.0 M NaCl-TPD (at 3-5 A_{260}/ml), allowed to extract for 30 minutes with periodic homogenization, and layered on a 10 ml cushion of 1.0 M sucrose-2.0 M NaCl-TPD. Centrifugation was performed at 60,000 rpm for 24 hours in the Beckman type 60Ti rotor. The extraction and centrifugation was performed twice to insure histone removal.

Separation of DNA-P and DNA-S: Centrifugation as above yielded a clear DNA pellet (DNA-S) underlaid by an opaque DNA-protein complex (DNA-P). The combined pellet was homogenized into TPD, and was allowed to swell overnight by dialysis against 4.0 l of TPD. After dialysis, a clear fraction and a diffuse DNA-protein precipitate was visually evident. This dialyzed mixture was collected and centrifuged at 10,000 rpm for 15 minutes in the Sorvall SS-34 rotor. The opaque DNA-P pellet was collected and the supernatant DNA-S poured off. The DNA-P pellet was washed several times with TPD until the supernatant A_{260} approached zero. This is a critical step, for incomplete washing leads to substantial contamination by DNA-S.

Extraction of Residual Protein from DNA-P: The DNA-P pellet (a complex of DNA and protein) was taken up in medium three[1], homogenized, and centrifuged at 60,000 rpm for 24 hours in the Beckman SW-60Ti rotor. The DNA pellet was collected from the bottom of the tube, purified, and used for further study.

Nick Translation of DNA: DNAs were labelled with ^{3}H-dCMP by the E. Coli

DNA Polymerase I repair reaction (8) using the method of Rigby et al. (9). The reaction mixture contained: 20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.05 mg/ml bovine serum albumin (Miles), 2.0 µM dATP, dGTP, dTTP (Sigma), 2.0 µM ^{3}H-dCTP (30 Ci/mmol, New England Nuclear), DNA Polymerase I, 2 units (ex. E.Coli MRE 600, Boehringer-Mannheim), 1 ng DNAase I (DPFF, Worthington), 1.0 µg DNA to be labelled, in a final volume of 100 µl. The reaction was run at room temperature for 1 hour, and then extracted with Tris-EDTA[1]-saturated phenol. Chromatography was done on a 0.7 x 20 cm column of Sephadex G-50 fine, preequilibrated with 0.1 x SSC[1], with the collection of 0.5 ml fractions. Aliquots were taken of each fraction, and counted in a toluene-Triton X-100 based liquid scintillation cocktail. Radioactivity appearing in the void volume identified the nick translated DNA. Specific activities ranged from 0.6 to 2.0 X 10^6 cpm/µg. The nick translated DNAs were made 0.24 M in NaCl and precipitated overnight with 2 volumes of absolute ethanol at -20° C.

DNA-DNA Reassociation: DNA-DNA reassociation was carried out essentially as described by Weintraub and Groudine (10). Driver DNAs were sheared using a Brownwill sonifier at maximum output in five second bursts to a total of five minutes, with intermissions to avoid overheating. Shearing in this manner generates random double-strand scission (11). Driver DNAs were sized on a 1.5% agarose gel as described (12), using ΦX-174RF-Hae III restriction fragments as markers. The average sizes of the sheared DNAs corresponded to 550-600 base pairs in length. For reassociation, the driver/tracer mass ratios exceeded 10,000 in all experiments. Aliquots were taken at zero time to assess the zero time S_1 nuclease resistance (ranging from 4-6%, but determined separately for each experiment). Aliquots were taken at the appropriate time intervals, and pipetted into S_1 nuclease buffer. To each vessel was added 1000 units of S_1 nuclease (Miles) and the digestion was carried out at 40° C for 40 minutes. The reaction mixture was spotted onto ion-exchange filter papers (DE-81, Whatman). The filters were washed five times with 0.5 M dibasic sodium phosphate, three times with deionized water, twice with absolute ethanol, and once with acetone. The filters were dried and counted in a Toluene-PPO scintillation cocktail in a Beckman LS-8100 liquid scintillation spectrometer.

Other Methods: All DNAs were purified essentially as previously published (13). Protein concentrations were estimated by the dye-binding method of Bradford (14). Spectrophotometric scans were carried out in a Pye-Unicam spectrophotometer equipped with a linear X-Y recorder. Concentrations for

purified DNAs were estimated using the relationship 20 A_{260}= 1 mg/ml for purified DNA. Purity of the DNAs was assessed by spectral criteria.

RESULTS

Although histones may maintain the intermolecular interactions which characterize the histone octamer in 2 M NaCl (15), the ionic interactions which maintain them on DNA are abolished, and hence they are completely extracted from DNA (7,16-18). However, after this extraction, a small percentage of nonhistone chromosomal proteins remains DNA-bound. The result of extraction of purified chicken reticulocyte chromatin with 2 M NaCl, with subsequent processing as described in Methods, is summarized in Table 1. Two different DNA fractions, differing both in properties and yield, are evident. Most of the DNA (ca. 99%) is rendered virtually protein-free, and solubilizes upon dialysis into 10 mM Tris-HCl, pH 8.0. A small percentage of the total DNA (ca. 1%) remains tenaciously protein-bound, displays a relatively high protein/DNA ratio, and is insoluble upon dialysis into 10 mM Tris-HCl, pH 8.0. The yield in DNA-P is relatively invariant from preparation to preparation using chromatin from the same tissue, but can vary substantially from different tissues of the same organism (unpublished observations), and this probably reflects different levels and organization of the tightly-bound nonhistone chromosomal proteins in different tissues. With respect to the extraction of reticulocyte chromatin, the small amount of protein found in DNA-P reflects a true protein-bound DNA complex, for reextraction with 2 M NaCl has no effect, and hence the complex found in DNA-P represents DNA bound by proteins which are truly resistant to 2 M NaCl extraction, rather than simply incomplete extraction during the first 2 M NaCl treatment.

The differences between DNA-S and DNA-P are illustrated in the spectrophotometric scans of Figure 1. DNA-S as prepared in Methods displays a scan

Table 1. Properties and yield of DNA-S and DNA-P.

Component	Protein/DNA[a]	Percent of Total DNA[b]
DNA-S	0.01	98.95
DNA-P	0.84	1.05

DNA-S and DNA-P were prepared as described in Methods. [a]after 2 M NaCl extraction, measured as described in Methods. [b]as determined after purification of the components, measured by comparison of A_{260}.

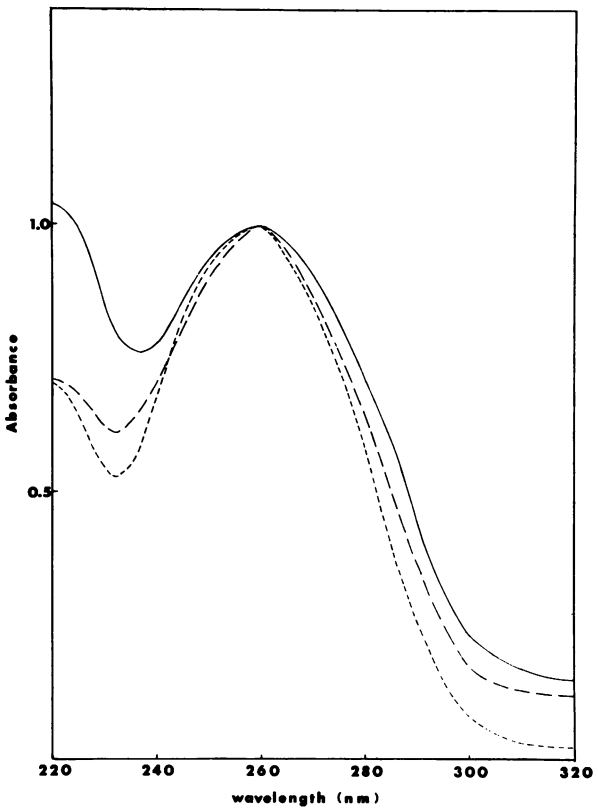

Figure 1. Spectrophotometric Scans of Segregated DNA Components. Scans were performed in 10 mM Tris-HCl, pH 8.0, 1.0 mM EDTA, using a Pye-Unicam spectrophotometer equipped with an X-Y recorder, as described in Methods. Symbols: DNA-S, short dashed lines; DNA-P, solid line; DNA-P after extraction with medium three, long dashed lines.

typical for protein-free DNA. DNA-P displays the displaced minimum typical for chromatin and also an increased A_{230}/A_{260} and A_{280}/A_{260} ratio, indicative of the substantial presence of proteins. However, upon extraction of the DNA-P complex with medium three (5 M urea - 3 M NaCl), the bulk of the residual protein can be extracted, and the scan of this DNA after protein extraction reverts to that of protein-free DNA; decreased minimum, and decreased A_{230}/A_{260} and A_{280}/A_{260} ratios. Hence, virtually all of the non-histone proteins responsible for this DNA segregation can be extracted from DNA in medium three, which rules out the rather remote possibility of covalent interaction between these proteins and DNA, with a resultant insol-

ubility, and further the requirement for the use of urea to extract these proteins illustrates the hydrophobic nature of their interaction with DNA.

Renaturation Kinetics

Having observed the phenomenon described above, it then became of interest to study the DNA sequences involved in this segregation. The purified DNAs of DNA-S and DNA-P are of essentially the same length, as determined by electrophoresis on a 1.5% agarose gel using phage λ-Eco RI restriction fragments as markers, and correspond to a range of between 5,000 and 20,000 base pairs in length. Some shearing is generated in the preparation of chromatin and subsequent processing. Indeed, this shearing is required to be able to separate DNA-P from DNA-S.

The main question of interest, however, is whether or not some sequence differences exist between DNA-S and DNA-P, i.e., is DNA-P a fully complete subset of the total sequences of unfractionated DNA, or is a noninclusive subset of these sequences selected? To answer this question, we prepared nick-translated, tritium labelled DNA probes from the purified DNA fractions for use in DNA-DNA reassociation. A potential problem could arise if artifacts were introduced during the nick translation procedure which could alter the renaturation properties of the probes. However, Mackey et al. (19) studied this question and found that a nick-translated viral probe displayed the same renaturation kinetics as that which had been labelled in vivo. This confirms the validity of this method, which has been used in a relatively large number of reassociation experiments.

As a calibration, we first prepared a radioactive probe of total unfractionated chicken DNA and measured its kinetics of reassociation with total chicken DNA as driver. The reassociation kinetics of this probe are presented in Figure 2. We find good agreement between this and other published measures of these kinetics (20,21). Repeated DNA sequences are found in the chicken genome, although at a lower proportion than higher eukaryotes (21), and these are reflected in the C_0t curve. We find that approximately 15% of the probe had reassociated prior to a C_0t value of 1, and approximately 30% prior to a C_0t of 100. The observed $C_0t_{\frac{1}{2}}$ value for this reaction is 316, with the reassociation being nearly complete at a C_0t value of 10,000, with only 4-5% of the probe being sensitive to S_1 nuclease digestion at this value of C_0t.

In direct contrast to these data is the renaturation curve of DNA-P in reassociation with unfractionated chicken DNA (Figure 3). Whereas the curve for the reassociation of total DNA indicates the presence of fast-reassociating

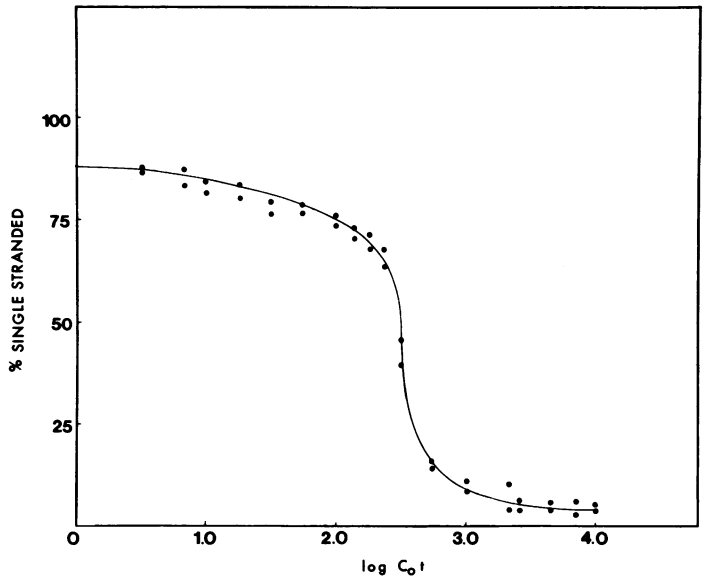

Figure 2. Reassociation Kinetics of Total Unfractionated DNA. DNA-DNA reassociation was performed as described in Methods. Specific activity of the probe was 6 X 10^5 cpm/µg. Duplicate assays were performed at each value of $C_o t$. Each assay received 600 cpm of labelled probe. Data is corrected for approximately 4% S_1 nuclease resistance at zero time.

sequences, these sequences appear to be completely absent in DNA-P as indicated by an apparent lack of reassociation in the lower values of $C_o t$. The observed $C_o t_{\frac{1}{2}}$ for this reaction is 160, or approximately one half of that observed for total unfractionated DNA. We interpret this to mean that in DNA-P, in addition to the depletion of repeating sequences, there is a concentration of a subset of unique sequences, leading to a more rapid reassociation. The reaction continues to a similar rate of completion as total unfractionated DNA at high $C_o t$. This data indicates that DNA-P is depleted of repetitive sequences, and contains a partial subset of total unique sequence DNA. The latter is also supported by the sharper reassociation transition seen in DNA-P.

The question then arises regarding the renaturation kinetics of DNA-S. Recalling that DNA-S is the DNA component rendered soluble by 2 M NaCl extraction and represents 99% of the recovered DNA from bulk chromatin, it

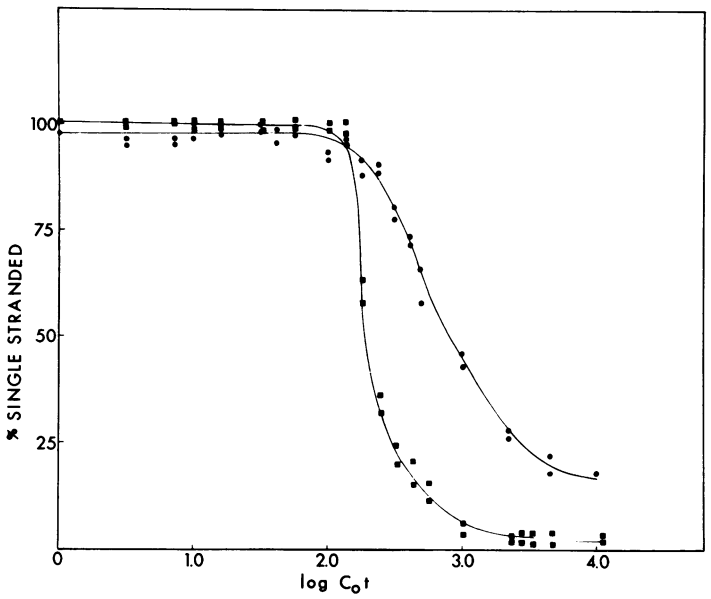

Figure 3. Reassociation Kinetics of DNA-P. The DNA probe was reassociated with total unfractionated chicken DNA (closed squares) and DNA-S (closed circles) as drivers. Specific activity of the DNA-P probe was 6.5 X 10^5 cpm/µg. Data is corrected for 4% S_1 nuclease resistance at zero time (for reassociation with total DNA), and 6% resistance at zero time (for reassociation with DNA-S). See legend to Figure 2 for further details.

would be expected that the renaturation kinetics of DNA-S would be very similar to those of total unfractionated DNA. The renaturation profile is presented in Figure 4. Again, the presence of fast reassociating components is obvious, and the overall profile is very similar to that presented for total unfractionated chicken DNA (Figure 2). The observed $C_0t_{\frac{1}{2}}$ for this reaction is again 316, or precisely that as measured for total unfractionated DNA. Hence, the renaturation kinetics of DNA-S are as expected and closely parallel those of total unfractionated DNA.

To investigate the relationship between DNA-S and DNA-P, we performed another reassociation experiment, cross-reassociating DNA-S and DNA-P, using DNA-S as driver, and DNA-P as tracer. The results of this experiment are presented in Figure 3, along with the reassociation kinetics of DNA-P in reassociation with total unfractionated DNA to facilitate comparison. Again the absence of rapid reassociation is seen. Further, the $C_0t_{\frac{1}{2}}$ value for

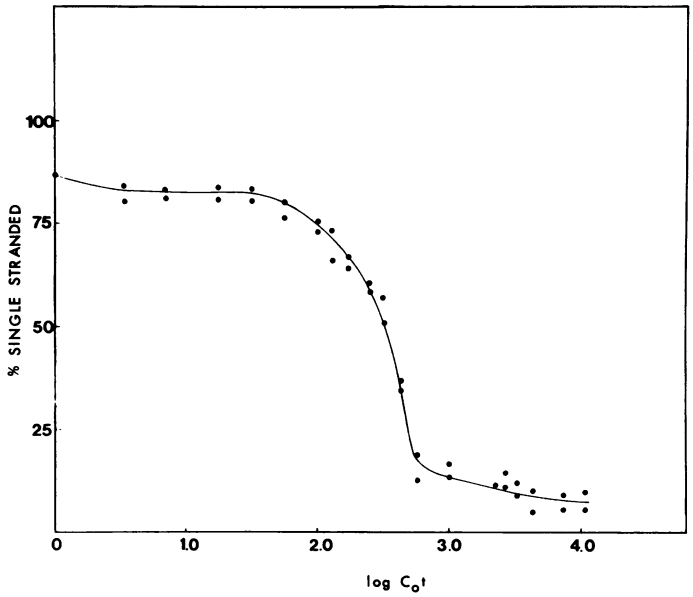

Figure 4. Reassociation Kinetics of DNA-S in Reassociation with Total Unfractionated Chicken DNA. Specific activity of the probe was 1.2 X 10^6 cpm/µg. Data is corrected for 6% S_1 nuclease resistance at zero time. See legend to Figure 2 for further details.

this reaction is 750, and the reaction reaches a much lower extent of completion at the C_0t value of 10,000, with approximately 20% of the probe remaining sensitive to S_1 nuclease digestion at this C_0t value. We interpret these findings to indicate that DNA-S is partially depleted of some DNA-P sequences, and nearly completely depleted of other DNA-P sequences. This duality of depletion can be explained on the following basis: during the process of preparation, DNA-S and DNA-P fragments are generated by random shearing. Since DNA-P is determined by the bound nonhistone chromosomal proteins, those DNA sequences most immediately contiguous with NHCP binding sites will be most strongly represented in DNA-P. Other DNA sequences more distal to the NHCP binding sites would then follow a more random distribution between DNA-S and DNA-P. It is possible that with greater shearing (thus generating shorter DNA fragments) it may be possible to isolate DNA-P sequences which are totally (or virtually so) depleted in DNA-S. In any event, we can be certain that at least a portion of the DNA-P sequences, as we have

Table 2. Summary of Reassociations.

Driver	Probe	Specific Activity of Probe[a]	Reiterated Sequences	$C_o t_{\frac{1}{2}}$	Completion
Total DNA[b]	Total DNA	6×10^5	+	316	94%
Total DNA	DNA-S	1.2×10^6	+	316	96%
Total DNA	DNA-P	6.5×10^5	-	160	96%
DNA-S	DNA-P	6.5×10^5	-	750	78%

DNA-DNA reassociation was performed as described in Methods. [a]Specific activity is defined as ^{3}H-dCMP counts per minute incorporated per microgram of DNA. [b]Total DNA is total unfractionated chicken DNA.

prepared them, are substantially depleted from DNA-S. The latter conclusion was substantiated by experiments on the distribution of globin gene sequences in DNA-S and DNA-P (7). It is evident that substantial sequence differences exist between DNA-S and DNA-P.

DISCUSSION

We have shown above that the DNA sequences retained by the tightly-bound nonhistone chromosomal proteins in chicken reticulocyte chromatin are a subset of the total unique sequence DNA. This implies that these proteins are nonrandomly distributed on DNA, with respect to sequence organization. The DNA sequences retained by these nonhistone proteins are largely, if not exclusively, of unique sequences, which may be important, particularly in view of the fact that, with few exceptions, eukaryotic messenger RNA appears to be transcribed from nuclear DNA of unique sequence (22). Other recent work from this laboratory (7) indicates that the DNA-P component from chicken reticulocyte chromatin (which actively synthesizes globin mRNA *in vivo*) shows a marked enrichment in the globin gene sequence, while the comparable DNA fraction from chicken liver (a non-erythroid tissue) does not. We hypothesize that DNA-P may be enriched in active gene sequences in general, with the expectation that those nonhistone chromosomal proteins which determine the DNA segregation in this *in vitro* fractionation may actually be regulatory proteins which control genetic readout of these coding sequences.

We cannot as yet make any definitive statements regarding the possible biological function(s) of these tightly bound nonhistone chromosomal proteins.

An interesting series of experiments (18,23,24) indicate that, for the metaphase chromosome, those proteins which are not dissociated in 2.0 M NaCl may be involved in the establishment and maintenance of the ordered metaphase chromosome structure. In this study, we are clearly not working with chromatin arrested at metaphase. Since this class of tightly-bound nonhistone chromosomal proteins is heterogeneous, it may well be that a multiplicity of biological functions are resident within this class.

Other workers studying protein-DNA interactions have reported that some DNA-binding nonhistone chromosomal proteins preferentially interact with repetitive DNA sequences within the rat genome (25,26). It is probable that the proteins under study in those experiments are not of the same general class as the tightly-bound nonhistone chromosomal proteins discussed in this report. We cannot totally exclude the possibility of the presence of very short repetitious DNA sequences by the methods employed in this study, but it would appear from the data presented that the tightly-bound nonhistone chromosomal proteins from chicken reticulocytes are exclusively associated with unique sequence DNA. The rat genome is known to display the pattern of interspersion of repetitious and unique sequences within DNA (27). After completion of our experimentation, Eden and Hendrick (28) reported that the organization of DNA sequences within the chicken genome is unusual in that approximately one-half of the DNA consists of very long single-copy regions of at least 17,500 nucleotides in length. Repeated DNA sequences do not occur within these long stretches of unique DNA. Thus it would appear that chicken DNA not only displays a different type of sequence organization than the rat, but also the tightly-bound nonhistone chromosomal proteins of the chicken reticulocyte display different DNA-binding preferences than those DNA-binding proteins studied in the rat. We do have some evidence (unpublished data) which indicates that as measured by <u>in vitro</u> DNA-binding activity, the tightly-bound nonhistones display marked preference for DNA-P (unique sequence) over DNA-S (a composite of reiterated and unique sequences).

Using chromatin reconstitution, it was reported (29) that the element controlling transcription of the globin gene in chicken reticulocytes reassociates with DNA prior to the histones. It is likely that this element is of the same general class of nonhistone chromosomal proteins as our tightly-bound proteins, which suggests that chromatin fractionation in the manner described in this report may be useful in the study of eukaryotic gene regulation. We have a particular interest in the question of sequence-specific interaction between the tightly-bound nonhistone chromosomal proteins and

DNA in the eukaryotic genome as it may relate to regulation of gene expression. The data reported herein are consistent with, and strongly suggestive of, sequence-specific recognition between the tightly-bound nonhistone chromosomal proteins of the chicken reticulocyte and DNA. This recognition may be related to the control of genetic readout.

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1. Abbreviations: EDTA, ethylene(dinitrilo) tetraacetic acid; NHCP, nonhistone chromosomal proteins; medium three, 5 M urea - 3 M NaCl in TPD; NET, 0.1 M NaCl - 1.0 mM EDTA in TPD; RSB, 0.1 M NaCl - 1.5 mM $MgCl_2$ in TPD; SSC, 0.15 M NaCl - 0.015 M NaCitrate; TPD, 10 mM Tris-HCl, pH 8.0 - 0.1 mM PMSF - 0.2 mM DTT; PMSF, phenyl methane sulfonyl fluoride; DTT, dithiothreitol.
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