

The effects of DNA intercalators on chromatin of chicken red blood cells --- differential extraction on nonhistone proteins

Wang Fan and Zhu Jingde

Shanghai Institute of Cell Biology; Shanghai Open Laboratory of Life Science, Academia Sinica, 320 Yue-yang Road. Shanghai 200031, China

ABSTRACT

Taking advantage of the effects on DNA secondary structure of two DNA-intercalators, ethidium bromide and chloroquine, we used each of them to treat nuclei from both mature erythrocytes and reticulocytes of chicken, as an alternative approach to study the relationships between DNA secondary structure, nuclear proteins and chromatin structure. We presented results of differential extraction of nuclear proteins from nuclei with DNA-intercalators, as well as preliminary characterization of these proteins. A 45kd protein is the major component in fractions extracted by both intercalators from nuclei from either mature erythrocytes or reticulocytes and seems to be a DNA-binding protein. Furthermore, from current concepts of functional aspects of DNA conformation and structural heterogeneity in chromatin and nuclear proteins, we have discussed both the significance of our results as well as technical aspects of this approach.

Key words: *DNA intercalators, ethidium bromide, chloroquine diphosphate, chromatin, nuclear proteins, heparin affinity chromatography.*

INTRODUCTION

The DNA component of eukaryotic genome can assume a wide spectrum of conformational variations which may provide an additional level of regulatory mechanisms over the gene expression [1]. Conformational variations, such as helix unwinding, strand unpairing. DNA bending, formation of cruciform structures and generation of left-handed Z DNA have been characterized biochemically *in vitro* [2]. They are

Correspondence: Zhu Jingde. Shanghai Institute of Cell Biology, Shanghai Open Laboratory of Life Science, Academia Sinica, 320 Yue Yang Road, Shanghai 200031, China.

manifested in both the sequence- and environmental- dependent manner [3]. DNA is found to be negatively supercoiled in all organisms investigated so far, except for thermophilic Archaeobacterium in which DNA is positively supercoiled [4]. The amazing feature of DNA negative supercoiling is the transmission over distance of its higher energy status within a close molecule or domain. Furthermore all detectable variants of DNA conformation are very sensitive to change of energy state created by alteration of the negative supercoiling density within a close DNA domain [5]. There are substantial evidences suggesting that most (if not all) DNA-binding reagents (including proteins) can change conformation of the interacting DNA region directly or indirectly by altering the density of the overall supercoiling of the close DNA domain or molecule [6-8].

DNA-protein interaction, especially the sequence-specific interaction is the key event in functional processes of genome, and attracts continuously great attention in current biological studies [9,10]. It is probably true that the real signaling form of the cis-elements *in vivo* available for trans-acting factors in all processes of genomic activities is the conformational variants of DNA. Therefore, in addition to efforts in characterizing binding properties of proteins at the primary structural level of DNA, researchers aiming to characterize the conformational-specific binding proteins have been actively undertaken. The proteins binding to Z-DNA or cruciform DNA binding proteins have been substantially purified from eukaryotic cells [11-12]. Hence, it has been generally believed that alteration of DNA conformation can influence the stability of DNA-protein complexes and *vice versa* [13-15].

In eukaryotic genome, chromatin is built up with DNA and heterogeneous proteins, and has tremendous capacity of varying its conformation at various hierarchies to meet different functional requirements in cell. The characterization of functionally active regions in the eukaryotic genome have been done in fair detail [11-17], and its less compact structure can be conveniently measured by assessing their sensitivity to DNase I. Transcriptionally active and potentially transcribable regions are usually more sensitive to DNase I digestion as much as 10-100 folds than the rest part of chromatin. At the 5'-end of those genes in nuclei, there are always one or a set of narrow regions (< 200 base pairs) with highest sensitivity to DNase I (DNase I hypersensitive sites, DHS) [16] as well as other DNA-reagents, both macromolecular and chemical [18]. The DHS at the 5'-end of genes are functionally important, as suggested by their close association with cis-control elements, such as promoters and enhancers for transcription [19, 20,]. It has been speculated that DHS may be the chromatin region holding unusual conformation of DNA, or associating with one or a specific subset of nonhistone proteins, or lacking of any core histones [16].

DNA-intercalators, such as ethidium bromide (EB) and chloroquine diphosphate (CQ) have strong effects on the DNA conformation by inserting their planar aromatic rings into between the base pairs of the neighboring nucleotides [21]. Most

striking are the unwinding of B-form of DNA and destabilization of Z-DNA [22]. Considering the differential DNase I sensitivity of DNA in nuclei reflecting the extent of compactness of DNA in chromatin, we argue that active regions of chromatin, especially DHS, are more accessible to DNA-intercalators and the stability of DNA-protein complexes at DHS are most likely to be altered. Hence, the proteins enriched at DHS, which presumably engage in the formation of DHS, would be preferentially extractable, if their affinity to DNA can be decreased by intervening of DNA-intercalators.

We used chicken red blood cell (RBC) and reticulocyte (Rec) as a model system to study the effects of DNA-intercalators (EB and CQ) on chromatin structure by assaying the protein profile extracted by EB or CQ from nuclei of both cells. We were able to show that in addition to HMG (high mobility group) 14 (probably HMG 17 too), certain proteins were also extractable. The proteins with molecular weight 45 and 32kd are two predominant ones and the former may be DNA-binding proteins.

MATERIALS AND METHODS

Nuclei preparation:

50ml blood was collected from slaughtered Leghorn hens into 150ml of cold 75mM NaCl/24mM EDTA, pH 7.0. After filtration through 2 layers of gauzes, red blood cells were pelleted by centrifugation with RPR12-2-632 rotor at 5000 rpm for 10 minutes at 4 °C in Hitachi high speed centrifuge. Cell pellet was washed once with 200 ml cold nuclei preparation buffer (10mM Tris-HCl, pH 7.4/10 mM NaCl/3mM MgCl₂/20% glycerol/2mM mercaptoethanol/0.1mM PMSF) and resuspended again in same solution at 4 °C. Cells were broken by gently shaking for 10 minutes in the presence of 0.4% TritonX-100 on ice. After over 95% cells were broken, nuclei were collected by 8000 rpm for 10 minutes at 4 °C with the same rotor. Nuclei were washed 3-4 times with nuclei preparation buffer. The final white nuclei pellet was resuspended in the same solution including 50% instead of 20% glycerol and stored at -50 °C. Nuclear concentration was estimated by measuring OD_{260nm} of nuclei suspension in 0.5M NaOH taking 1 OD_{260nm} as 35 μg of chromatin.

Reticulocytes (Rec) were obtained from phenylhydrazine (Sigma)-induced anemic hen. 5mg phenylhydrazine in 0.9% NaCl/0.1% folic acid/0.01% Vitamin B₁₂ / kg body weight of hens per day was injected for 5 days. On the 7th day, the hen was killed and circulating blood was collected. Normally over 95% of red blood cells are Rec. Nuclei preparation from Rec was performed as from RBC.

Protein extraction from nuclei with DNA-intercalators:

Nuclei were suspended in cold nuclei preparation buffer at a concentration of 15 mg/ml. Various amounts of EB or CQ (Sigma) were mixed with nuclear suspension to reach a final concentration of 5-25 mM. After 3 hours of incubation with continuous shaking on ice nuclei were pelleted at low speed, and the supernatant was reserved for further analysis. A no-intercalator control was carried out in parallel. The remaining nuclei were resuspended

Effects of DNA intercalators on chromatin of chicken RBC

(40mg/ml) in nuclear preparation buffer and digested with DNase I (200 μ g/ml) at 37°C overnight. Before adding an equal volume of 2X sample buffer (4% SDS, 10% β -mercaptoethanol, 20% glycerol / 0.125M Tris-HCl, pH6.8), centrifugation of DNase I digests was taken and pellets were discarded. After treating the sample in boiling water for 2 minutes, 30 μ l per well was applied for SDS-polyacrylamide gel electrophoretic analysis.

SDS-polyacrylamide gel electrophoresis of proteins:

Protein content in each extraction was either directly analyzed by a 5-20% SDS-polyacrylamide gel electrophoresis [23] or further fractionated before analysis. 2% trichloroacetic acid (TCA) fractionation of proteins was pursued as follows: 100% cold TCA was added dropwise into protein extracts to a final concentration of 2% TCA and incubated on ice for 30 min. Precipitated proteins collected by high speed centrifugation at 4°C were washed three times with acidified acetone (acetone: 0.1 M HCl=7: 1) and once with acetone. After air-drying, pellet was redissolved in sample buffer (2% SDS /5% β -mercaptoethanol/ 10% glycerol / 62.5 mM Tris -HCl, pH 6.8) and incubated in boiling water for 2 minutes before electrophoresis. Heparin- affinity chromatography of the extracts was performed at 4-10°C. The extracts were changed to column running buffer (50 mM Tris -HCl, pH 7.9 at 4°C/ 1 mM EDTA/ 1 mM DDT/ 12.5 mM MgCl₂/ 0.1 mM PMSF/ 20% glycerol) plus 0.1 M KCl, before chromatography. The protein fraction eluted with 0.4 M KCl were collected for electrophoretic study.

A micro-2D gel electrophoresis was carried out as follows: The isofocusing of 1 μ g 2% TCA precipitated proteins was done at a pH gradient from pH 3 to 8. The 2nd dimensional analysis is pursued on a 7% SDS-polyacrylamide gel [24]. After electrophoresis, the gel was either stained with silver [25], or Commassie Blue G [26]. Protein size markers (Dong-Feng Biochemical Manufacture or Sigma) and calf thymus histone markers (Sigma) are used in parallel.

The electrophoretic graphs are densitoscanned and analyzed by a computer program processing system (CID-100) for image analysis developed by Zhang Yaoyuan [submitted]. The percentage of proteins of interests within the extract is estimated by comparing the paper-weight of the peak related.

RESULTS

EB extracted profile of proteins from RBC nuclei:

At a low osmotic condition, nuclei may break due to osmotic shock and nuclear proteins may leak out from nuclei. On the other hand, high osmotic condition created by higher ionic strength tends to weaken the affinity of most nuclear proteins to DNA. Consequently, proteins can leak out and nuclei may break too. With the precaution to avoid potential complexity in interpretation of results concerning the effects of DNA- intercalators on the protein- extractability, we used a buffer containing very low concentration of salt, i.e. 10 mM NaCl, but with high osmotic stress created by high concentration of glycerol (20%) to prepare nuclei, and used as the

basic solution for treating nuclei with DNA intercalators. In addition, the buffer also contains 3 mM MgCl₂ to stabilize nuclei. In no-intercalator control, few proteins were extracted at low quantity (0.1 mg/ml) from RBC nuclei at a nuclei concentration of 15 mg/ml. Incubation of nuclei with a common DNA-intercalator, EB, at 5-20mM concentration, led to the release of a subset of nuclear proteins from RBC nuclei (Fig. 1). Total amount of extracted proteins increased as much as 5-25 fold

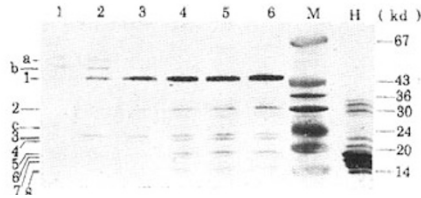


Fig. 1

Protein profile of the extracts from RBC nuclei with or without EB. 15 mg/ml RBC nuclei were incubated in nuclei preparation buffer with various amounts of EB. No-EB control (lane 1) was set in parallel. 20 μ l of supernatants from each extracts were analyzed in a 5-20% SDS-PAGE gel system and silver-stained. Lanes 2-6, extracts by EB at concentrations of 5, 10, 15, 20 and 25 mM respectively. Calf thymus histones (Sigma, lane H) and 7 protein size markers (Sigma, lane M) were used. Their kd value are indicated on right side of the photograph. On the left side, a, b and c, as well as 1-8 indicate the protein-bands of interests. Protein size markers are: bovine albumin: 67, ovalbumin: 43, glyceraldehyde -3-phosphate dehydrogenase: 36, carbonic anhydrase: 30, trypsinogen: 24, trypsin inhibitor: 20, and α -zinc talbumin: 14 kd.

depending on EB concentration. With a high sensitive silver-staining technique, We were able to detect proteins in each fraction without any concentrating process. Comparing the protein profile of EB extracts (lane 2-6) with no-EB control (lane 1), we have observed two different effects of EB on the extractability of nuclear proteins. First, the proteins became less extractable in the presence of EB, (a to c, on the left side of Fig. 1) and were completely absent in the extract by 10 mM EB (lane 3, Fig. 1). Second, the proteins were preferentially extracted by EB (1-8 in Fig. 1). Along with the increase of EB concentration (5-20 mM), the extractability of those proteins increased accordingly. The most striking one was protein 1 (MW= 45kd) whose amount increased as much as 20-70 fold. Furthermore, this protein occupied over 90% in quantity in EB extracts when EB concentration was over 10 mM. From their mobility in the SDS-PAGE and with calf thymus histones as reference, it seems that band 3 is HMG14, and band 5 may be HMG17 (N.B. in this condition, HMG17 co-migrates with H3). Bands 5-8 were core histones H3, H2B, H2A and H4, respectively. In the presence of 15 mM EB, core histones started to release but their release did not increase accordingly with the increase of EB concentration, and even became less when EB concentration is 25 mM (compares lanes 4-6). This finding may suggest there is an

upper limit of core histones that can be extracted from nucleus by EB, and it is likely to be set by the proportion of chromatin which is more sensitive to DNase I as well as other DNA reacting reagents, including EB. 10mM EB may be sufficient for saturating those chromatin regions. Alternatively, the further EB intervening may significantly raise the stability of this complex. This was also supported by results in Fig. 3.

In order to demonstrate more clearly the result shown in Fig.1, we densitoscanned Fig.1 as shown in Fig.2. Panel A shows the scannogram over each lane

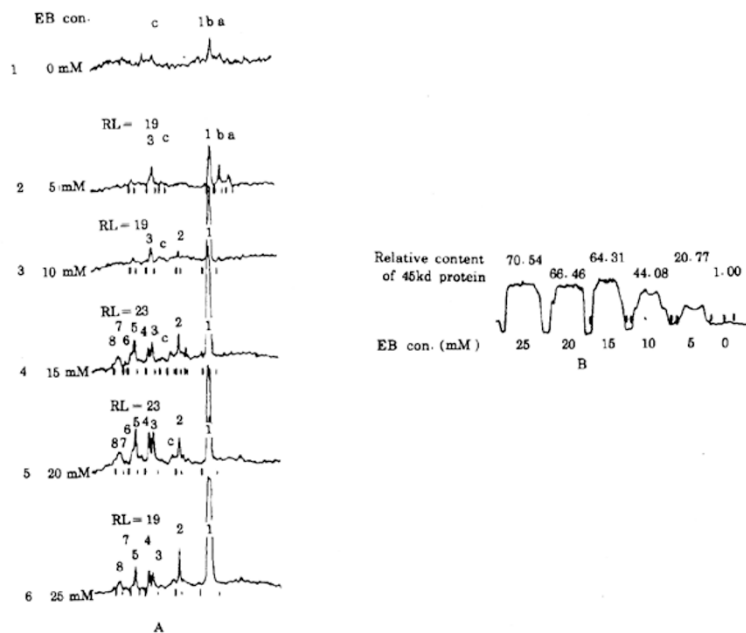


Fig. 2 Densitoscanning-analysis of EB extracts of RBC nuclei. Panel A shows the scannogram of lanes 1 to 6, Fig.1, vertically, and panel B shows the scannogram of 45kd protein horizontally across the lanes 1 to 6. The peaks corresponding to the protein bands of interests are marked by a, b, c and 1 to 8 respectively. The peaks in panel B have been quantitated by measuring the mass of paper under each peak, and calculate individually, by taking no-EB control as 1. The figures above each peak represent the relative value over the no-EB control value

of lanes 1-6 in Fig. 1, whereas panel B is the scanogram of 45kd protein band across lanes 1-6. Panel B shows that with the increase of EB concentration, more 45 kd protein were released from nuclei upto 70.54 fold than the control.

We have also assessed the extractability of 45kd protein in nucleus by 25 mM

EB treatment. The nuclear pellet after 25 mM EB extraction were intensively digested by DNase I (200 $\mu\text{g/ml}$) overnight, and released proteins were analyzed in parallel with EB extract as well as total nuclear proteins. In Panel A, Fig. 3,

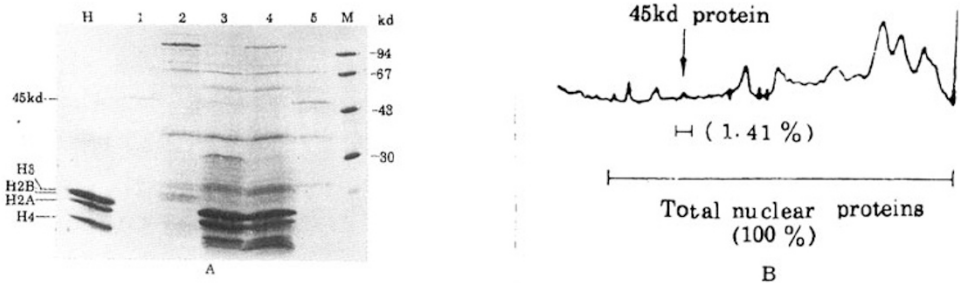


Fig. 3 Comparison of protein profile in EB or CQ extracts of RBC nuclei with the remaining nuclei.

Panel A, After 25mM EB or CQ extraction (lanes 1 and 5: EB and CQ extracts respectively), RBC nuclear pellets were resuspended in nuclear extraction buffer (40mg/ml) and digested by DNase I (200 $\mu\text{g/ml}$) overnight. The released fractions (lane 2, EB, and lane 4, CQ,) and a no-intercalator-control (lane 3) were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue G 250. Lanes: H. calf thymus histone markers, M. Sigma protein size markers as before.

Panel B, densitoscanning of lane 3 in panel A, 45kd protein peak is indicated by arrow, and the percentage of 45kd protein within nuclear protein population is calculated by comparing the paper weight of 45kd peak to the integrate of all peaks.

comparing lane 1 (EB extract) with lane 2 (the remaining pellet) and lane 3 (total nuclear proteins), we observed that majority of 45kd protein in nucleus was extractable by 25 mM EB. In addition, core histones hardly released out after such intensive DNase I digestion (lane 2). In agreement with this finding, we found that no more, even less core histones became extractable when EB concentration was over 10 mM (Fig. 1).

In order to quantitate 45kd protein in nucleus, we used a computer programme for image analysis to analyze the densitoscanning graph from the lane 3 in panel A of Fig. 3, which is shown in panel B in Fig. 3. 45kd protein occupies approximately 1/70 of total nuclear proteins, suggesting its abundancy in nucleus.

CQ releases a similar group of proteins from nuclei as EB

To ascertain an intercalating mechanism for EB effects on extractability of nuclear proteins, we treated the nuclei with another DNA-intercalator, CQ, and compared its extracted protein-profile with that of EB (Fig. 4). In addition to the two major proteins (45kd and 32kd) extracted by EB, CQ can extract more proteins.

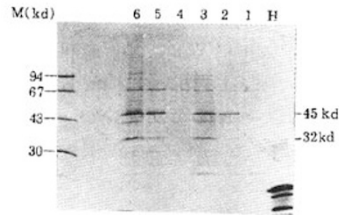


Fig. 4

Comparative analysis of protein profiles extracted with EB or CQ from RBC or Rec nuclei. 20 μ l of extracts from RBC nuclei (lanes 1-3) and Rec nuclei (lanes 4-6) were subjected to a 18% SDS-PAGE analysis and stained with Commassie blue G 250. Lanes 1 and 4 are controls, lanes 2 and 5 are 25mM EB extracts, lanes 3 and 6 are 25mM CQ extracts. Protein size markers are phosphorylase b: 94kd, bovine albumin: 67, ovalbumin: 43, and carbonic anhydrase: 30 kd.

This result may indicate that besides the common intercalating effects implemented by both EB and CQ, there were differences in mechanisms or efficiency between EB and CQ in interacting DNA. Results in panel A, Fig. 3, also showed the differences between EB and CQ extract.

Differences between RBC and Rec in their extracting pattern of proteins by same DNA-intercalators from nuclei

Reticulocyte (Rec) is at a stage prior to the mature red blood cells on the erythrocytic differentiation pathway. Rec is different from RBC in many aspects of the genomic functions. In Rec, both DNA replication and transcription are actively undertaken, but neither occur in RBC. Hence, it is desirable to ask whether there is any difference in EB or CQ extracting profiles of nuclear proteins between RBC and Rec. Using phenylhydrazine to destroy mature erythrocytes and stimulate erythrocytic regenerating mechanism on erythropoietic pathway in bone marrow, is a convenient method to obtain Rec from anemic hens. With this approach, we were able to obtain Rec preparation with less than 5% RBC contamination. Fig. 4 also shows the EB or CQ extracting profile of proteins from Rec nuclei (lanes 5 and 6). Although, a more heterogeneous proteins occurred in Rec extracts, the general pattern of proteins extracted by either EB or CQ between RBC (compares lanes 2 and 5) and Rec (compares lanes 3 and 6) were very similar. Considering the detectability of the method used, we can not conclude simply from the data (Fig. 4) that the detectable differences in protein profile extracted by DNA-intercalators relate to difference of genome function between RBC and Rec.

Further characterization of the intercalator-extractable proteins

For further characterization, it is necessary to concentrate the proteins of interests. TCA precipitation is frequently used for concentrating and fractionating proteins. In the presence of a higher concentration of TCA (20- 25%), most of the

proteins, if not all, are precipitable. While with a lower concentration of TCA, it is possible to fractionate the proteins by differential precipitation. We have tested various concentration of TCA (2- 25%) to precipitate EB or CQ extracts and found that a higher concentration of TCA caused insolubility of the precipitated proteins from EB extracts. With 2% TCA, most proteins of interests (including 45kd and 32kd) were precipitated. Except for HMG 14, we used 2% TCA to concentrate (fractionate) the proteins extracted by DNA- intercalators (Fig. 5). Since the

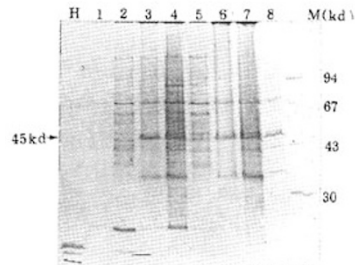


Fig.5

SDS-PAGE analysis of 2% TCA precipitable proteins of EB or CQ extracts. The extracts shown in Fig. 4 were precipitated with 2% TCA. Precipitated proteins from 500 μ l extracts were analyzed on a 18% SDS-PAGE and stained with Com-massie blue G 250. Lanes: 1 and 8, 20 μ l of RBC control and 25 mM CQ extracts of Rec nuclei, respectively; 2-7, 2% TCA pre cipitates from 500 μ l of control, 25 mM EB or CQ extracts of RBC nuclei as well as the control. 25 mM EB or CQ extracts of Rec nuclei, respectively. Protein size markers are same as one in Fig. 4.

extracts have been fractionated rather than simply concentrated for probably 25 fold, protein patterns of EB or CQ extracts were different from the original (Fig. 4). However, the more prominent ones were still 45kd and 32kd. In addition, the difference between control and the EB or CQ extracts, of both RBC and Rec nuclei was still obvious.

To analyze the EB-extractable protein profile at a finer level, we used a micro 2-D electrophoretic method to make a detail comparison of EB extracts and no-EB control (Fig. 6). Focus our attention on proteins bigger than 30kd, a 7% SDS-PAGE was used for the second dimension electrophoresis. In addition to 45 kd and 32kd proteins, a 94kd protein was also EB extractable. They were all acid proteins with pI:4-5 for 45kd, 4-5.5 for 32kd, and 5-6 for 94kd protein. Whereas, few dots, around 45 to 67 kd with pI 7.0, missing in panel B, indicating that EB made them less extractable. Furthermore, there were also other minor proteins EBextractable and some proteins which were indifferent to EB extraction as indicated by their approximately equal amounts in both panel A and B, Fig. 6.

It is commonly believed that proteins having regulatory roles in gene expression should have significant affinity to DNA and rather higher affinity to the cis-elements of genes. If proteins, extractable by EB or CQ are enriched at DHS, then these protein fractions should have, at least, some proteins with higher affinity to DNA.

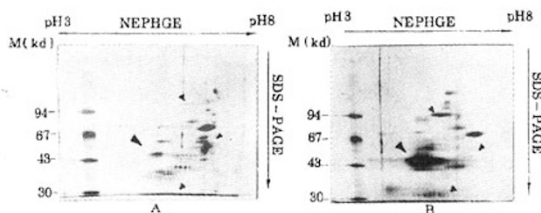


Fig. 6

2D-electrophoretic profiles of 2% TCA precipitates of 25 mM extracts and no-EB control of the RBC nuclei. 2% TCA precipitate of 50 μ l (1 μ g) of control (panel A) and 25 mM EB (panel B) extracts of RBC nuclei were compared in a micro-2D- electrophoretic system. The first dimension is a pH 3-8 nonequilibrium polyacrylamide gel electrofocusing, and the second dimension is a 7% SDS- PAGE electrophoresis. The proteins were revealed by silver -staining. The filled triangles are used to pinpoint the groups of dots which differ in density between two panels.

We used heparin affinity chromatography to fractionate EB and CQ extracts. Our purpose is: First, heparin is an acid polysaccharide, holding a similar ionic feature as DNA, and this column has been commonly used for preliminary fractionation and purification of DNA sequence-specific binding proteins [27]. The proteins eluted by a higher salt condition, such as 0.4 M KCl are usually enriched with proteins recognizing regulatory regions for RNA polymerase II genes [20]. Second, we may enrich the proteins of interests, and, at the same time remove DNA- intercalators, which may interfere the functional characterization of proteins by DNA-protein binding assay. In Fig. 7, it is shown that 0.4 M KCl eluant of heparin chromatography

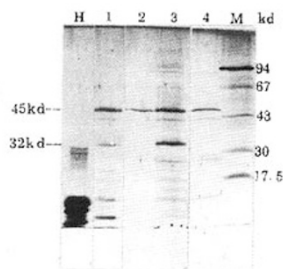


Fig.7 Heparin affinity chromatography of EB or CQ extracts. 500 μ l of extracts was applied to heparin column (Dong-feng Biochemical Manufacture) chromatography in column solution containing 0.1 M KCl. 20 μ l extracts (lane 1, 25 mM EB and lane 3, 25 mM CQ) as well as 20 μ l among 500 μ l of 0.4 M KCl eluents (lane 2, EB, and lane 4, CQ) were analyzed on a 18% SDS- PAGE and silver-stained.

(lane 2 and 4) contained much less heterogeneous proteins than the original (lanes 1 and 3). 45kd protein was almost the sole protein left from EB extract (lane 2); in CQ fraction, although there were more proteins left (lane4), but 45kd protein is still predominant. The belief that proteins in 0.4 M KCl eluant are likely DNA-

binding proteins was also based upon our preliminary data from DNA-protein binding assay (unpublished results).

DISCUSSION

The most common way to obtain protein factors which may play an important role in gene transcription and replication is to extract the protein from nuclei with buffers consisting of higher ionic strength than the physiological one and fractionate the extracts later [27]. The rationale behind is that hydrophilic interaction is one major force for DNA-protein interaction. Weakening the hydrophilic interactions between proteins and nuclear DNA by an increased concentration of salt in nuclear extraction buffer will promote the proteins of interests dissociating from nuclear DNA. In this report, we presented a potentially alternative approach for extracting proteins whose interaction with DNA may be weakened by the intervening of DNA-intercalating reagents. It may be true that DNA-intercalators, EB or CQ, significantly alter DNA conformation directly by intercalating effect, and affect affinity of proteins to DNA. Thus, these proteins would dissociate from DNA and become extractable. Furthermore, structural heterogeneity of genome *in vivo* may be able to form the basis for nonrandom distribution of input DNA intercalators along chromatin, just like DNase I preferentially attacks at the chromatin regions (DHS) with loosely packed structure, enriching with the positively regulatory factors and functioning in gene transcription. Hence, we expect the fraction released from the DNA-intercalator treated nuclei is enriched with the positively regulatory factors. Relevant to this notion, we do have evidences that some proteins extracted by CQ or EB from Rec and RBC nuclei are DNA binding proteins and having substantial specificity to the expressing genes, i. e. the chicken β -globin genes (unpublished observations). Furthermore, the result from heparin chromatograph (Fig. 7) suggested that 45kd protein and some other proteins in 0.4M KCl eluant from heparin chromatography, having higher affinity to DNA, may be trans-acting transcription factors [27]. Finally, in agreement with this notion, a recently published report has demonstrated that a CQ extract from Hela nuclei contains a 67kd protein, acting as a trans-regulatory protein for the transcription of *c-fos* gene [28]. On the other hand, the released proteins may play a role in formation of DHS. In the absence of those proteins, DHS may alter in position or density. In this context, we have obtained supporting evidence from mapping DHS in EB treated RBC and Rec nuclei (unpublished results). Alternatively, the changes of DNA conformation at DHS or nearby regions by the intervening of EB can also change DHS pattern.

In addition to HMG14 and probably HMG 17 [29], which are two common structural proteins with relatively high affinity to DNA and probably preferentially associating with active nucleosomes [30], we have been able to show that at least two rather abundant proteins, i. e. 45kd and 32kd, are both EB and CQ-extractable.

Considering the similar pattern of the major proteins extracted by either EB or CQ (Fig. 4), a common mechanism, namely, intercalating effect, is probably involved. However, since CQ extracted more heterogeneous nuclear proteins than EB, and core histones have different response to EB and CQ treatment (Fig. 3), we can not exclude the possibility of the involvement of some other mechanisms.

We noticed that there are difference in the protein extraction pattern by DNA intercalators between Schroter et al's [29] and ours. They did not detect 45 kd and 32 kd proteins, and HMG 14 and 17 were prominent components. In our hands, nuclei were broken by DNA intercalators using their extracting buffer, whereas in our buffer condition, nuclei were still intact which is essential for further experimentation. Although we are not sure at this point if the 20% glycerol in our buffer alone makes such drastic effect, we believe that some differences in chemical composition of their buffer [29] and ours (such as 3 mM $MgCl_2$) is influential too.

It is intriguing to find that some proteins became less extractable in the presence of DNA-intercalators. This may suggest that some types of proteins-DNA interaction become strengthened by DNA-intercalators. Apparently, final conclusion on this issue needs further study.

The nature of 45kd protein is a very important question to address, since it is the most predominant one to be extracted by either EB or CQ. It is the sole component detected by SDS-PAGE electrophoresis in 0.4 M KCl eluant of heparin chromatography of EB extract, and the overwhelming major one in the CQ equivalent fraction (Fig. 7). It is possible that 45kd protein is a DNA binding protein with higher affinity to DNA than HMG 14 and 17, as suggested by the fact that the latter two proteins are absent in 0.4 M KCl eluant. We have substantial evidence that 45kd protein is DNA-binding protein by the Western-blotting-DNA binding assay [30] with higher affinity to the adult chicken β -globin gene than plasmid DNA (unpublished results). But its abundancy in nucleus, as much as 1.4%, of total nuclear protein (Fig. 3) argues against the contention that it is a trans-acting factor involving specifically in globin transcription. The explanations for its functional role may be either a common transcription factor, or the protein (such as the nuclear matrix proteins) to demarcate structural and functional domains in genome [31,32]. At the moment, we are not able to tell whether it is an important protein in gene expression in chicken erythroid cell. But it will be both informative and worthwhile to study the DNA-binding specificity of 45kd protein on a set of upstream regulatory elements of chicken genes in future.

Other intercalator extractable proteins, such as 32kd and 94kd proteins (Fig. 6) may not be DNA-binding proteins due to their absence in 0.4 M KCl eluents of heparin chromatography. But it is possible for them to play an important role in protein-protein interaction which occurs in all genome functional processes as protein-DNA interaction does. It is apparent that to answer this important question requires further experimentation with the update both *in vivo* and *in vitro* trans-

cription assays [33] as well as DNA-protein interaction assays [27,28].

ACKNOWLEDGMENT:

This work is supported by the National Science Foundation of China.

Thanks due to Mr. Chang Yaoyuan for computer analysis of the protein electrophoretic data, to Mrs. Son Qiu bao for her technical assistance for Fig. 6, and to Dr. Yao Zhen for critical reading of this manuscript.

REFERENCES:

- [1] Rich A. Right-handed and left-handed DNA conformational information in genetic material. Cold Spring Symp Biol 1980 ; 47: 1-12.
- [2] Rich A, Nordheim A, & Wang AHJ. The chemistry and biology of left-handed Z-DNA. Ann Rev Biochem 1984 ; 53 : 791-846.
- [3] Peticolas WL, Wang Y, & Thomas GA. Some rules for predicating the base-sequence dependence of DNA conformation. Proc Natl Acad Sci USA 1988 ; 85 : 2579 -2583.
- [4] Modal M, Mirambeau G Forterre P Reiterey W & Duguet M. Positively supercoiled DNA exists *in vivo*. Nature 1988 ; 21 : 256-258.
- [5] Wang JC, Peck L J, & Becherer K. DNA supercoiling and its effects on DNA structure and function. Cold Spring Harbor Symp Quant Biol 1983 ; 47 : 85-91.
- [6] Pabo C, & Sauer RT. Protein-DNA recognition. Ann Rev Biochem 1986 ; 53 : 293- 321.
- [7] Ebralidse KK, Grachev SA, and Mizabekov W. A high basic histone H4 domain bound to the sharply bent region of nucleosomal DNA. Nature 1988; 331 : 365-367.
- [8] Shurey DU, & Parker HS. 1986, Bending of promoter DNA on binding of heat shock transcription factor. Nature 1986 ; 323 : 459-461.
- [9] Dynan WS, & Tjian R. 1985, Control of eukaryotic messenger RNA synthesis by sequence-specific DNA binding proteins. Nature 1985 ; 316 : 774-448.
- [10] Sassone - Corsi P, & Borrelli E. Transcriptional regulation by trans-acting factors. TIG 1986 ; 2: 215 -219.
- [11] Elborough K, & West SC. 1988 . Specific binding of cruciform DNA structures by a protein from human extracts. Nucl Acids Res 1988; 16(9) : 3603 -3615.
- [12] Azorin F, & Rich A. Isolation of Z-DNA-binding proteins from SV- 40 minichromosomes, evidence for binding to the viral control region. Cell 1985 ; 41 : 365-374.
- [13] Reynolds WF, and Gottesfeld JM. 5S rRNA gene transcription factor III A alters the helical configuration of DNA. Proc Natl Acad Sci USA 1983 ; 80 : 1862 -1868.
- [14] Piette J, and Yaniv M. 1986 , Molecular analysis of the interaction between an enhancer binding factor and its DNA target. Nucl Acids Res 1986 ; 14 (24): 9595 - 9611.
- [15] Bryan PN, and Folk WR. Enhancer sequences responsible for DNase I hypersensitivity in polyoma virus chromatin. Mol Cell Biol 1986; 6(6) : 2249- 2252.
- [16] Eissenberg JC, Cartwright IL, Thomas GH, & Elgin, SCR. Selected topics in chromatin structure. Ann Rev Genetics 1985 : 19 : 485-535.

- [17] Pedson DS et al. Core particle , fibre and transcriptional active chromatin structure. *Ann Rev Cell Biol* 1986 ; 2: 117 -147.
- [18] Cartwright IL, Hertzberg RP, Dervan PB, & Elgin SCR. Cleavage of chromatin with methidiumpropyl -EDTA, iron (II). *Proc Natl Acad Sci USA* 1983 ; 80 : 3213 - 3217.
- [19] Felsenfeld G, Emerson BM, Jackson PD, Lewis CD, Hesse JE, Lieber MR, & Nickol JM. Chromatin structure near an expressed gene. In: Poste G, & Cook ST eds. 'New Frontier in the Study of Gene Function', Pleum, -Smith Kline & French Laboratories Research Symposia Series 1986.
- [20] Jean KT & Khoury G. The mechanistic role of enhancer elements in eukaryotic transcription. *Bioassays* 1988 ; 8 (4): 104- 107.
- [21] Neidle S, & Abraham Z. 1984, Structural and sequence-dependent aspects of drug intercalation into nucleic acids .*CRC Critical Reviews in Biochemistry* 1984; 17(10) :73 -121.
- [22] Pohl FM, Jovin TM, Bear W, & Holbrook JJ. 1972, Ethidium bromide as a cooperative effector of a DNA structure. *Proc Natl Acad Sci USA* 1972 ; 69: 3805- 3809.
- [23] Lammili UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970 ; 227 : 680- 683.
- [24] Wray Y, Boulikas T. Wray VP. & Hancock R. Silver staining of proteins in polyacrylamide gels . *Analytic Biochemistry* 1981 : 118 : 197-203 .
- [25] Neukirchen RO, Schlosshauer B. Baars S. Jackle H. & Schwaz. U. Two-dimensional protein analysis at high resolution on a microscale. *J Biol Chem* 1982: 24 : 15229-15234.
- [26] Holbrook B, & Leaver AG. A procedure to increase the sensitivity of staining by Commassie brilliant blue G250 - perchloric acid solution. *Analytic Biochemistry* 1976; 75: 634 -636.
- [27] Grave BJ, Johnson PF. & Macknight S. Homologous recognition of a promoter domain common to MSV LTR and HSV TK gene. *Cell* 1986 : 44: 565- 576.
- [28] Schroter H, Shaw PE. & Nordheim A. Purification of intercalator-released p67, a polypeptide that interacts specifically with the *c-fos* serum response element. *Nucl Acids Res* 1987 ; 15(24) : 10145 -10158.
- [29] Schroter H, Maier G. Ponsting H, & Nordheim A. DNA intercalators induce specific release of HMG14, HMG17 and other DNA-binding proteins from chicken erythrocyte chromatin. *The EMBO J* 1985 : 4 (13B): 3867-3872.
- [30] Gershoni JM. Protein-blotting : developments .and perspectives. *TIBS* 1985 ; 103-106 .
- [31] Gasser SM, & Lammili UK. 1987. A glimpse at chromosomal order. *TIG* 1987; 3: 16-22.
- [32] Loc PV, & Straling WH. The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. *The EMBO Journal* 1988 ; 7(3): 655- 664.
- [33] Wasylyk, B. Transcription elements and factors of RNA polymerase B promoters of high eukaryotes. *CRC Critical Reviews in Biochemistry* 1988 : 23 (2): 77- 120.

Received 7-11-1988

Accepted 23 -1-1989.