

THE IN VITRO RECONSTITUTION OF NUCLEOSOME AND ITS BINDING PATTERNS WITH HMG1/2 AND HMG14/17 PROTEINS

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ABSTRACT

Using atomic force microscopy (AFM), the dynamic process of the in vitro nucleosome reconstitution followed by slow dilution from high salt to low salt was visualized. Data showed that the histone octamers were dissociated from DNA at 1M NaCl. When the salt concentration was slowly reduced to 650 mM and 300 mM, the core histones bound to the naked DNA gradually. Once the salt concentration was reduced to 50 mM the classic "beads-on-a-string" structure was clearly visualized. Furthermore, using the technique of the in vitro reconstitution of nucleosome, the mono- and di- nucleosomes were assembled in vitro with both HS2core (-10681 to -10970 bp) and NCR2 (-372 to -194 bp) DNA sequences in the 5'flanking sequence of human b-globin gene. Data revealed that HMG 1/2 and HMG14/17 proteins binding to both DNA sequences are changeable following the assembly and disassembly of nucleosomes. We suggest that the changeable binding patterns of HMG 14/17 and HMG1/2 proteins with these regulatory elements may be critical in the process of nucleosome assembly, recruitment of chromatin-modifying activities, and the regulation of human b-globin gene expression.

Keywords: *atomic force microscopy, HMG proteins (HMG1/2 and HMG14/17), human b-globin gene, in vitro reconstitution, nucleosome.*

INTRODUCTION

In eukaryotic cells, gene expression is not determined solely by DNA itself. It is influenced by proteins that package DNA into chromatin. However, the molecular mechanisms by which chromatin plays its dynamic role in regulating gene activity are poorly understood. Much is now known concerning the structure of the nucleosome, the fundamental repeating unit of eukaryotic chromatin. The physical properties of nucleosome depend on solution conditions such as ionic strength and divalent-ion concentration as well as on histone-modifi-

cation state. Recent evidences show that acetylation or methylation of histone proteins can alter the patterns of gene activity. In living cells the process of nucleosome assembly and disassembly, recruitment of chromatin-modifying activities, and regulation of gene activities is very complicated and dynamic.

In our previous studies the chromatin folding patterns in chicken erythrocytes were revealed by AFM. At the first level of DNA packing, our data showed that an extended beads-on-a-string structure (width of ~15-20nm, height of ~2-3 nm for each individual nucleosome) could be consistently observed. Furthermore, superbeads (width of ~40 nm, height of ~7 nm) arranged with irregular distance along the extended beads-on-a-string were demonstrated[1]. In addition, the structure of the nucleosome core particle of chromatin in chicken erythrocytes was further revealed by using AFM. The ~146 bp of DNA wrapped twice around the core histone octamer

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Abbreviations: HMG, High Mobility Group proteins; AFM, Atomic Force Microscopy; HS2 core, the core DNA sequence of DNaseI HyperSensitive site 2; NCR2, Negative Control Region 2; EMSA, Electrophoresis Mobility Shift Assay.

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were clearly visualized. Both the ends of entry/exit of linker DNA were also demonstrated[2].

In an attempt to gain further insight into the dynamic processes of the *in vitro* nucleosome reconstitution, the linker histones-depleted oligo-nucleosomes were prepared from chicken erythrocytes. The process of the *in vitro* nucleosome reconstitution followed by slow dilution from high salt to low salt was imaged with AFM. Once the salt concentration had been reduced to 50 mM, the classic "beads-on-a-string" structure was clearly visualized (width of ~18nm, height of ~2-4 nm for each individual nucleosome).

Furthermore, using the technique of the *in vitro* reconstitution of nucleosome, the mono- and di-nucleosomes were assembled with both the core DNA sequence of DNase I hypersensitive site II (HS2core DNA sequence, -10681 to -10970 bp, ~290 bp) in LCR and NCR2 (-372 to -194 bp, ~178bp) DNA sequence in 5' flanking sequence of human β -globin gene. HS2 is an important control element within β -globin LCR. It functions as a typical enhancer. While, NCR2 is a silencer in the 5' flanking sequence of human β -globin gene[3]. It is our further aim to examine whether HMG proteins (HMG1/2 and HMG14/17) can bind to both the HS2 core and the NCR2 DNA fragments presented in different states (either in the naked DNA or in the *in vitro* reconstituted nucleosomal DNA). HMG proteins are abundant chromosomal non-histone proteins. It has been shown that HMG proteins may play an important role in the structure and function of chromatin. However, their role in the regulation of eukaryotic gene transcription remains to be established. We show here that HMG 1/2 proteins are able to bind to both the naked HS2core and NCR2 DNA sequences. While, these proteins are unable to bind to both HS2core and NCR2 DNA sequences assembled *in vitro* into nucleosomes with the core histone octamers transferred from the linker histones-stripped oligo-nucleosomes. In contrast, HMG14/17 proteins are unable to bind to both naked HS2core and NCR2 DNA sequences, whereas they are capable of binding to the nucleosomes assembled *in vitro* with HS2 core and NCR2 DNA fragments. It seems that the binding patterns of HMG1/2 and HMG14/17 proteins to both the distal and proximal regulatory elements of human β -globin gene are changeable following the assembly and disassembly of nucleosomes. Results presented here might provide some important clues for both the process of *in vitro* reconstitution of nucleosomes and the biological functions of

HMG proteins (HMG1/2 and HMG14/17 proteins) in the structure of chromatin and in the regulation of globin gene expression.

MATERIALS AND METHODS

Preparation of the linker histones -stripped oligo-nucleosomes

Nuclei were prepared from chicken erythrocytes and processed as described by Lutter[4]. These nuclei were then digested with micrococcal nuclease (40 units/ml) in the presence of 1 mM CaCl₂ for 5 min at 37 °C. The reaction was stopped by adjustment to 2 mM EDTA. The solution is centrifuged for 10 min (3000 x g) at 4 °C. The pellet was resuspended and lysed by pipetting gently up and down in 2.5 ml of lysis solution (10 mM Tris-HCl pH 7.5, 0.2 mM EDTA and 0.2 mM PMSF). The solution was kept on ice for at least 30 min and was shaken gently to release the chromatin into solution. Then, the solution was centrifuged again for 10 min (3000 x g) at 4 °C. The supernatant was adjusted to 0.65 M NaCl and then applied to a sepharose 4B column (2cm x 80cm) that had been equilibrated with 0.65 M NaCl, 5mM Tris-HCl (pH7.5), 0.2 mM 2-mercaptoethanol. Fractions containing A₂₆₀ absorbing material were collected. Small portions of representative column fractions were electrophoresed on 18% (w/v) acrylamide and 0.1% (w/v) SDS to examine the presence of histone H1 or H5.

The in vitro reconstitution of nucleosome and AFM analysis

The nucleosome reconstitution was performed according to the method of Drew et al. [5]. The solution containing the linker histones (H1 and H5)-stripped oligo-nucleosomes was adjusted to 1M NaCl and 1 mM PMSF, followed by slow dilution from high salt (1M NaCl) to low salt (50 mM NaCl), and tapping mode images were obtained on a nanoscope III AFM (Digital instruments, Santa Barbara, CA), using nanoprobe silicon tips, at a scan rate of 1.5 Hz.

The in vitro reconstitution of nucleosomes with both the labeled HS2core and NCR2 DNA fragments

The H1 and H5-depleted oligo-nucleosomes were prepared from the nuclei of chicken erythrocytes. The labeled HS2core and NCR2 DNA fragments were reconstituted into nucleosomes with the core histone octamers transferred from the H1 and H5-depleted oligo-nucleosomes[6]. Samples were electrophoresed on 5 % acrylamide gel and autoradiographed.

Gel mobility shift assay

HMG14/17 and HMG1/2 proteins were prepared from the nuclei of chicken erythrocytes according to the method of Goodwin et al [7]. The protein concentration was determined by Bradford's method [8]. Both HS2core DNA fragment (-10681 to -10970 bp) and NCR2 DNA fragment (-372 to -194 bp) in 5' flanking sequence of human β -globin gene were prepared with restriction enzymes and labeled with γ -³²P-

ATP. Gel mobility shift assay was performed according to the method described previously[9]. The HMG proteins were incubated with the DNA probe in 25 μ L reaction mixture (25 mM Tris-HCl, pH 7.9, 0.05 mM EDTA, 0.5mM DTT, 0.5 mM PMSF and 5% glycerol) on ice for 45 min with addition of enough polydI-polydC as non-specific competitor. The binding of HMG proteins to the DNA probe was resolved by 6% non-denaturing polyacrylamide gel.

RESULTS

Identification of the linker histones-stripped oligonucleosomes isolated from chicken erythrocytes

To study *in vitro* nucleosome reconstitution, the linker histones-stripped oligonucleosomes were prepared from chicken erythrocytes. Small portions of representative column fractions were electrophoresed on 18% (w/v) acrylamide and 0.1% (w/v) SDS to examine whether the linker-histones (H1 or H5) were stripped. Data revealed that linker histones (H1 or H5) could not be detected in samples examined (Fig 1, lanes 1-2 and 4-6). Then, the linker histones-depleted oligo-nucleosomes (containing \sim 15 nucleosomes) were used for *in vitro* reconstitution tests.

*Visualization of the dynamic process of *in vitro* nucleosome reconstitution by AFM*

In order to reveal the process of *in vitro* nucleosome reconstitution, the samples of *in vitro* nucleosome reconstitution at different concentrations of NaCl were prepared and imaged by using AFM. Results demonstrated that the histone octamers were dissociated from DNA

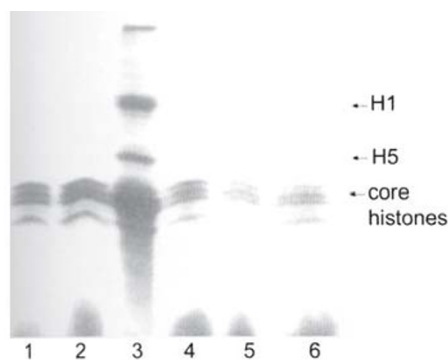


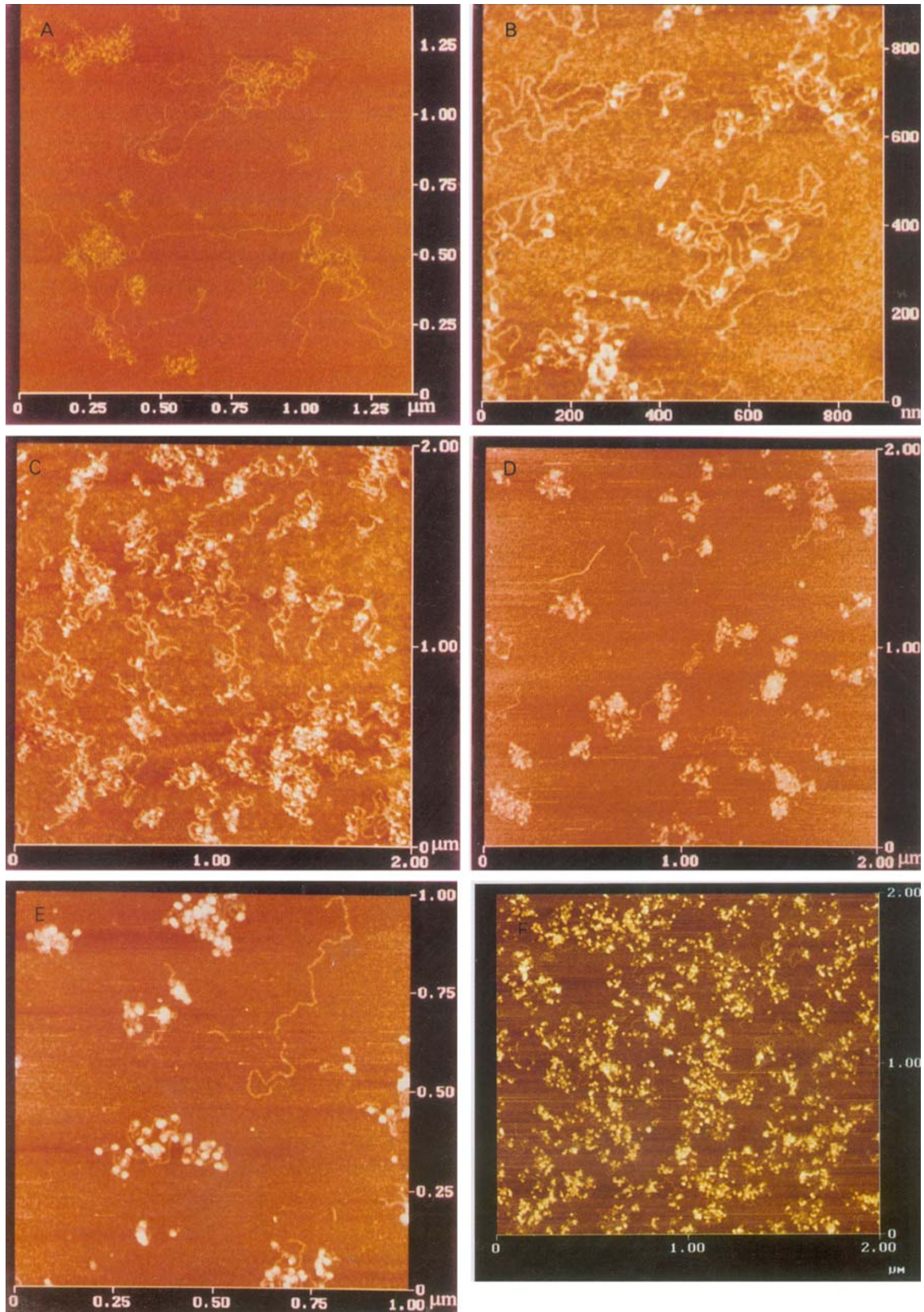
Fig 1. Identification of histone proteins in the linker histones-stripped oligo-nucleosomes. Small portions of representative column fractions were electrophoresed on SDS-PAGE. Lanes 1-2 and 4-6, The linker-histones (H1 and H5) could not be detected in the samples examined; lane 3, total histones isolated from chicken erythrocytes as a marker.

at 1M NaCl (Fig 2A). Furthermore, when the concentration of NaCl was slowly reduced to 650 mM and 300 mM, the histones were capable of binding to the naked DNA again (Fig 2B and Fig 2C). Then, the oligo-nucleosome fibers were folded into the compact fibers when the concentration of NaCl was reduced to 100 mM (Fig 2D). Furthermore, these fibers were further loosened when the salt concentration was diluted to 50 mM (Fig 2E). The classic "beads-on-a-string" structure was clearly visualized (height of \sim 2-4 nm, width of \sim 18 nm for each individual nucleosome). Results demonstrated that the *in vitro* reconstituted nucleosomes did not differ in morphology from native ones[1].

*Identification of *in vitro* reconstitution of nucleosomes with the HS2core DNA fragments*

The Locus Control Region (LCR) is located at 6-22kb upstream of human β -globin gene. It is characterized by four DNase I hypersensitive sites (HS1, HS2, HS3 and HS4). HS2 is a typical enhancer. The unlabeled HS2core DNA fragment (-10681 to -10970 bp, \sim 290 bp) was used to reconstitute *in vitro* into nucleosomes with the core histone octamers transferred from the H1 and H5-depleted oligo-nucleosomes. The *in vitro* reconstituted mono- and di-nucleosomes could be visualized by using AFM (Fig 3A and Fig 3B). The thin arrows point to the reconstituted mono-nucleosomes and the thick arrows indicate the reconstituted di-nucleosomes. The length of the used HS2core DNA fragment was only 290 bp. Therefore the linker DNA between the reconstituted di-nucleosomes was very short, while the DNA sequence flanking the reconstituted mono-nucleosomes was quite long.

Furthermore, the mono- and di-nucleosomes were assembled *in vitro* with the γ - 32 P-ATP labeled HS2core DNA fragment (Fig 4, lanes 2-4, bands A and B). The band of the reconstituted di-nucleosomes (Fig 4, lanes 2-4, band A) was very similar to that of the native di-nucleosome from chicken erythrocytes (Fig 4, lane 5, band A). However, the band of the *in vitro* reconstituted mono-nucleosomes (Fig 4, lanes 2-4, band B) migrated significantly slower than that of the native mono-nucleosome from chicken erythrocytes (Fig 4, lane 5, band C). The reason is that the length of the labeled HS2core DNA fragment (\sim 290 bp) is longer than that of the native mono-nucleosomal DNA (\sim 146 bp) isolated from chicken erythrocytes. Data further confirmed that the *in vitro* reconstitution of nucleosome was successful.



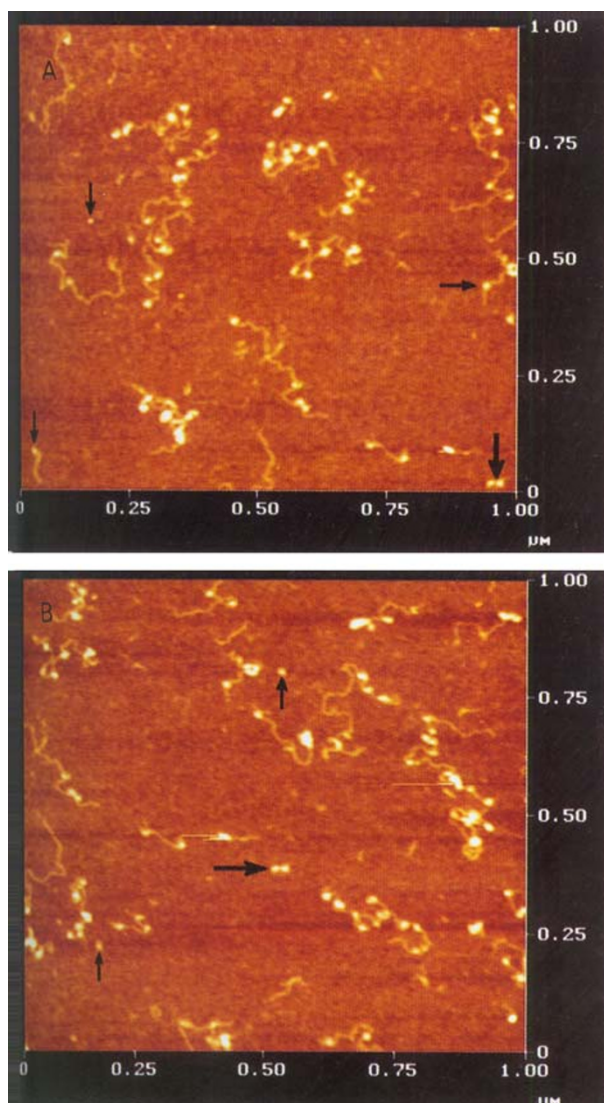


Fig 3. (A and B). AFM images of the *in vitro* reconstitution of nucleosomes with the unlabeled HS2core DNA fragments. The *in vitro* reconstituted mono- and di-nucleosomes could be visualized. Thin arrows point to the reconstituted mono-nucleosomes and thick arrows indicate the di-nucleosomes.

◁ **Fig 2.** AFM images of the dynamic process of the *in vitro* nucleosome reconstitution. Samples of the *in vitro* nucleosomes reconstitution at different concentrations of NaCl were prepared and imaged with AFM. (A) The histones octamers were dissociated from DNA at 1M NaCl. (B) And (C) the core histones bound to DNA gradually when the concentration was slowly reduced to 650 mM and 300 mM. (D) The fibers were folded into the compact fibers at 100 mM NaCl. (E) The beads-on-a-string structure could be visualized at 50 mM NaCl. (F) The linker histones-stripped oligo-nucleosomes as a control.

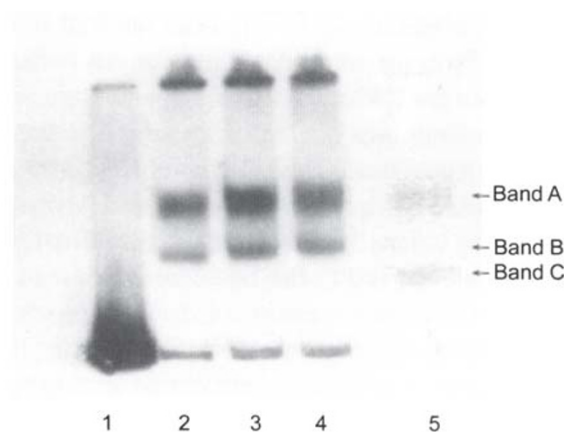


Fig 4. Identification of *in vitro* reconstitution of nucleosomes with the labeled HS2core DNA fragments. Lane 1, the naked labeled HS2core DNA probe; lanes 2-4, the *in vitro* reconstitution of nucleosomes: the ratios of the linker histones-stripped oligo-nucleosomes to labeled HS2core DNA are 100:1, 150:1, and 200:1 respectively; lane 5, the native mono-, di-nucleosomes prepared from chicken erythrocytes were stained with EB as markers.

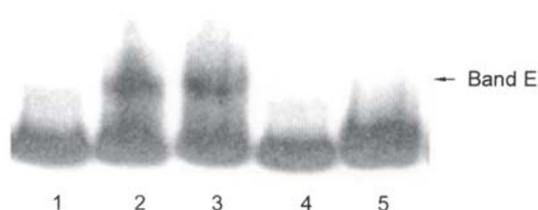


Fig 5. Gel mobility shift assay of HMG proteins binding to the naked HS2 core DNA sequence. Lane 1, labeled HS2core DNA fragment without HMG proteins; lanes 2 and 3, labeled HS2 core DNA fragment with 1.5 and 2 mg of HMG (1/2) proteins respectively; lanes 4 and 5, labeled HS2 core DNA fragment with 1.5 and 2 mg of HMG (14/17) proteins respectively.

The binding of HMG proteins to HS2core DNA sequence in LCR

In order to investigate the binding of HMG proteins (HMG1/2 and HMG14/17) to the HS2core DNA sequence presented in different states (either in the naked DNA or in the *in vitro* reconstituted nucleosomal DNA), Gel mobility shift assays were performed. When the naked HS2core DNA fragment was used as a probe we demonstrated that no band could be detected by addition of HMG 14/17 proteins (Fig 5, lanes 4-5). While, One shift band (band E) could be revealed by addition of HMG1/2 proteins (Fig 5, lanes 2-3, band E). Data showed that HMG1/2 proteins could bind to the naked HS2core

DNA sequence, whereas the HMG14/17 proteins couldn't. Furthermore, both bands 'A' and 'B' (mono- and di-nucleosomes represented respectively) could be visible when the HS2core DNA fragments were reconstituted *in vitro* into nucleosomes (Fig 6, Lanes 1). Moreover, a new band (band D) was observed by addition of HMG14/17 proteins and the band A was also retarded (Fig 6, lane 3), indicating that HMG14/17 proteins are able to bind to the HS2core DNA sequence reconstituted *in vitro* into nucleosomes. While, no change could be detected by addition of HMG1/2 proteins, indicating that these proteins are unable to bind to the *in vitro* reconstituted nucleosomes (Fig 6, lanes 4-5). It seems that dynamic control of human β -like globin gene expression requires the regulated assembly and disassembly of the architectural component- enhancer DNA complex.

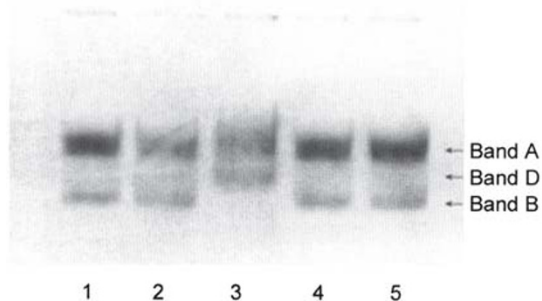


Fig 6. Gel mobility shift assay of HMG proteins binding to the nucleosomes reconstituted *in vitro* with labeled HS2core DNA fragment. Lane 1, the nucleosomes reconstituted *in vitro* with labeled HS2core DNA fragment; lanes 2 and 3, the nucleosomes reconstituted *in vitro* with labeled HS2core DNA fragment. 5 and 10 μ g of HMG14/17 proteins were added respectively; lanes 4 and 5, the nucleosomes reconstituted *in vitro* with labeled HS2 core DNA fragment. 5 and 10 μ g of HMG1/2 proteins were added respectively.

The binding of HMG proteins to NCR2 DNA sequence in 5' flanking DNA sequence of human b-globin gene

Next, we were interested in testing the binding of HMG proteins (HMG1/2 and HMG 14/17) to the NCR2 (a silencer, -372 to -194 bp, ~178 bp) in the 5'flanking sequence of human b-globin gene[3]. The mononucleosomes were also reconstituted *in vitro* with this DNA fragment (Fig 7, lanes 1-2, band F). In addition, data further showed that HMG 1/2 proteins could bind to the naked NCR2 DNA sequence (Fig 8, lane 1, band G),

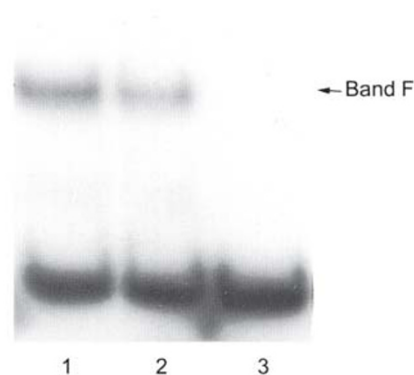


Fig 7. Identification of *in vitro* reconstitution of nucleosomes with labeled NCR2 DNA fragments. Lanes 1 and 2, *in vitro* reconstitution of mono-nucleosomes; the ratios of linker histones-stripped oligonucleosomes to labeled NCR2 DNA are 200:1 and 100:1 respectively; lane 3, naked labeled NCR2 DNA probe.



Fig 8. Gel mobility shift assay of HMG proteins (HMG1/2 and HMG14/17) to naked NCR2 DNA sequence (-372 to -194bp) in the 5'flanking sequence of human b-globin gene. Lane 1, labeled NCR2 DNA fragment with 1 μ g of HMG1/2 proteins; lane 2, labeled NCR2 DNA fragment without HMG proteins; lane 3, labeled NCR2 DNA fragment with 1 μ g of HMG 14/17 proteins

whereas HMG14/17 proteins couldn't (Fig 8, lane 3). In contrast, HMG14/17 proteins were capable of binding to the mono-nucleosome reconstituted *in vitro* with NCR2

DNA fragment (Fig 9, lane 1, band H), whereas HMG1/2 proteins were unable to bind to it (Fig 9, lane 2). These results further confirmed that the binding of HMG proteins (HMG1/2 and HMG14/17) to the tested distal and proximal regulatory elements in the 5'flanking sequence of human b-globin gene is dynamic following the assembly and disassembly of nucleosome. We suggest that the dynamic binding patterns of HMG proteins to the regulatory elements might be related to the regulation of the human b-globin gene expression.

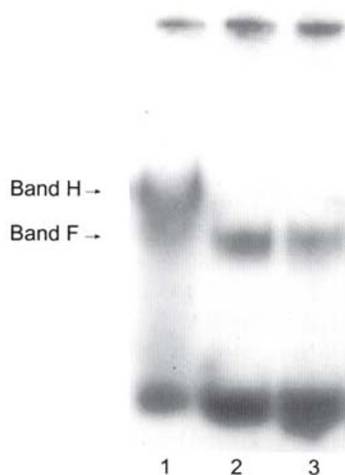


Fig 9. Gel mobility shift assay of HMG proteins (HMG1/2 and HMG14/17) binding to the mono-nucleosomes reconstituted in vitro with labeled NCR2 DNA fragment. Lane 1, mono-nucleosome reconstituted in vitro with labeled NCR2 DNA fragment, 5 μ g of HMG14/17 proteins was added; lane 2, mono-nucleosome reconstituted in vitro with labeled NCR2 DNA fragment, 5 μ g of HMG1/2 proteins was added; lane 3, the mono-nucleosome reconstituted in vitro with labeled NCR2 DNA fragment.

DISCUSSION

The orderly progression of most DNA-related activities such as transcription, replication, recombination and repair involves changes in the structure of DNA and in the organization of chromatin fiber. To understand how a long extended DNA folds into a chromosome is one of the most important problems in biology. All eukaryotic chromosomes consist of a regularly repeating protein-DNA complex called the nucleosome[10]. It is traditionally considered to be the fundamental unit of chromatin structure. The X-ray crystal structure of the complete nucleosome core particle was resolved at 2.8 \AA resolution, providing a detailed view of the protein and DNA organization[11].

Recently, the atomic force microscopy (AFM) has

been used for exploring the structure of chromatin[12, 13]. The technique of AFM has several advantages over more classical imaging techniques like EM. It allows imaging of biological macromolecules and structures in ambient conditions, without staining or shadowing, in air or under liquids. Therefore, AFM can image biological macromolecules under conditions close to their native condition. It provides more clearly images of the structure of chromatin than that attained by electron microscopy. Using the atomic force microscopy, we have previously revealed the structure of the nucleosome core particle of chromatin in chicken erythrocytes: the \sim 146bp of DNA wrapped twice around the core histone octamer were clearly visualized. Both the ends of entry /exit of linker DNA were also demonstrated. The dimension of the nucleosome core particles is \sim 1-4 nm in height and \sim 13-22 nm in width[2].

Nucleosomes and chromatin fibers are dynamic, not inert. Their dynamic behavior is essential for chromatin function. Most DNA in vivo is packaged in nucleosomes, and many DNA sequences are critical for biological regulation and so must be accessible to regulatory proteins at appropriate times. What are the principles that guarantee that regulatory proteins will have access to their DNA target sequences when necessary?

In an attempt to gain further insight into the dynamic processes of in vitro nucleosome reconstitution, the linker histones-depleted oligo-nucleosomes were prepared from chicken erythrocytes. The dynamic process of in vitro nucleosome reconstitution followed by slow dilution from high salt to low salt was visualized by AFM. Once the salt concentration has been reduced to 50 mM, an extended beads-on-a-string structure could be clearly visualized (height of \sim 2-4 nm, width of \sim 18 nm for each individual nucleosome).

Gene-Specific transcriptional switches are thought to be generated through the dynamic assembly and disassembly of transcription factor-enhancer DNA complex. The high mobility group proteins (HMG proteins) are a set of non-histone proteins in the nuclei of all higher eukaryotes. However, the biological functions of these proteins are poorly understood. They are generally considered to function as architectural elements that modify the structure of DNA and chromatin to generate a conformation for regulating various DNA-dependant activities. HMG 14/17 proteins are the only nuclear proteins known to specifically recognize the generic structure of the 146 bp-nucleosome core[14-16]. They are

modestly enriched in the actively transcribed gene relative to the inactive gene[16-19]. It was known that HMG 14/17 proteins could enhance the replication and transcription when the chromatin was used as template[20].

We previously revealed that HMG proteins (HMG1/2 and HMG14/17) could specifically interact with the regulatory elements in the 5'flanking sequence of the human e-globin gene and suggested that these proteins might participate in the regulation of this gene's expression[21]. In addition, Our previous studies demonstrated that HMG1/2 proteins were able to bind to a negative regulatory region NCR1 (-618 to -373 bp) in the 5'flanking sequence of human b-globin gene. Using scanning tunneling microscopy (STM) we revealed that the binding of HMG1/2 to NCR1 could bend this DNA fragment to form a circular structure with a linear tail[22]. Therefore, we tend to conclude that the changes of the local DNA conformation induced by HMG1/2 proteins may play an important role in regulating the expression of human b-globin gene.

In order to further investigate the interactions between HMG proteins (HMG1/2 and HMG14/17 proteins) and the HS2core DNA sequence in LCR, the in vitro reconstitution of nucleosomes with the γ -³²P-labeled and unlabeled HS2core DNA fragments was carried out. Results showed in EMSA that HMG 1/2 proteins could bind to the naked HS2core DNA sequence. While, HMG14/17 proteins could not. Furthermore, HMG14/17 proteins were capable of binding to the mono- and di-nucleosomes reconstituted in vitro with HS2core DNA sequence. In contrast, HMG1/2 were unable to bind to them. Meantime, the NCR2 (-372 to -194 bp) in the 5'flanking sequence of human b-globin gene was also assembled in vitro into mono-nucleosomes. Data showed that the binding patterns of HMG1/2 and HMG14/17 proteins to the NCR2 presented in different states (either in the naked DNA or in the in vitro reconstituted nucleosomal DNA) were consistent with that of HS2core sequences. We suggest that the changeable binding patterns of HMG1/2 and HMG14/17 proteins with the regulatory elements may be critical in the process of nucleosome assembly, recruitment of chromatin-modifying activities, and the regulation of human β -globin gene expression.

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