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A method for evaluating nucleosome stability with a protein-binding fluorescent dye

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ABSTRACT

Nucleosomes are extremely stable histone–DNA complexes that form the building blocks of chromatin, which accommodates genomic DNA within the nucleus. The dynamic properties of chromatin play essential roles in regulating genomic DNA functions, such as DNA replication, recombination, repair, and transcription. Histones are the protein components of nucleosomes, and their diverse modifications and variants increase the versatility of nucleosome structures and their dynamics in chromatin. Therefore, a technique to evaluate the physical properties of nucleosomes would facilitate functional studies of the various nucleosomes. In this report, we describe a convenient assay for evaluating the thermal stability of nucleosomes *in vitro*.

1. Introduction

In eukaryotes, chromatin functions to accommodate the genomic DNA within the nucleus [1]. The fundamental repeating unit of chromatin is the nucleosome. In the nucleosome, 145–147 base pairs of DNA are left-handedly wrapped 1.65 turns around the histone octamer, which is composed of two each of histones H2A, H2B, H3, and H4 [2]. The nucleosome is generally inhibitory for replication, repair, recombination, and transcription of genomic DNA. Thus, the nucleosome dynamics that ensures the genomic DNA functions has emerged as the central player in the epigenetic regulation of genes. All core histones are post-translationally modified, by acetylation, methylation, phosphorylation, ubiquitylation, and other modifications [3,4]. In addition, non-allelic isoforms of histones H2A, H2B, and H3 have been found as histone variants [5–11]. These histone modifications and histone variants confer diversity to the structure and stability of nucleosomes, and function as epigenetic components of chromatin. Therefore, a method for directly evaluating nucleosome stability would facilitate analyses of chromatin function.

Förster/fluorescence resonance energy transfer (FRET) has been used to evaluate nucleosome stability [12–14]. In the FRET method, fluorescent dyes are attached to DNA ends and/or histone surfaces in the nucleosome, and the nucleosome dynamics are studied by the FRET signal between two fluorescent dyes. This method has been widely used to study the effects of DNA sequence variations [15], DNA methylation [16,17], DNA damage [18], histone acetylation [12,19,20], and nucleosome-binding proteins [21,22] on nucleosome stability. The nucleosome assembly, disassembly, and remodeling dynamics with or without histone chaperones and chromatin remodelers have also been studied by the FRET method [23–26]. Thus, the FRET-based nucleosome stability assay is an excellent method to study nucleosome stability and dynamics. However, in the FRET assay, fluorescently labeled DNA and/or histones must be prepared. Therefore, a more convenient assay for evaluating nucleosome stability would be useful to study the dynamic properties of nucleosomes containing distinct histone variants, histone mutants, histone modifications, and DNAs.

Nucleosome stability has conventionally been studied by other spectroscopic methods, such as ultraviolet and circular dichroism measurements [27–29]. These assays evaluate the nucleosome stability based on the changes of the DNA conformations in the nucleosomal and/or naked DNAs. Therefore, these conventional spectroscopic methods are excellent for monitoring the DNA structures in nucleosomes; however, they are not suitable for studying histone disassembly from nucleosomes.

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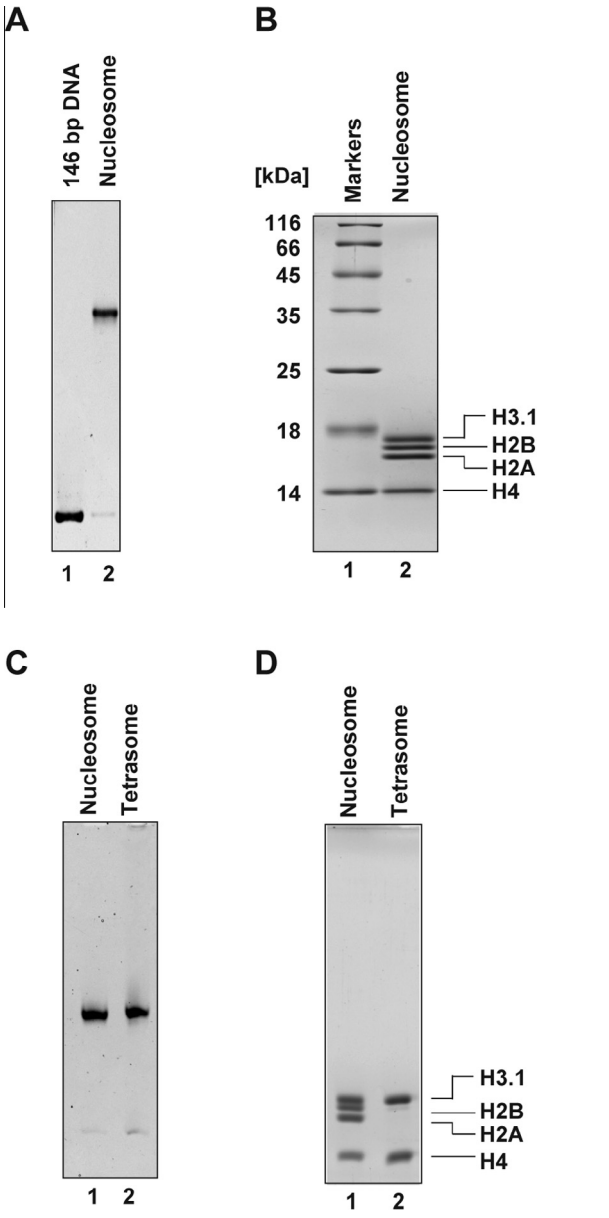

Fig. 1. Reconstitution and purification of the nucleosome and the tetrasome. (A) The purified nucleosome was analyzed by 6% non-denaturing polyacrylamide gel electrophoresis with ethidium bromide staining. Lanes 1 and 2 indicate the 146 base-pair DNA and the nucleosome, respectively. (B) The nucleosome was analyzed by 18% SDS–polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining. Lanes 1 and 2 indicate molecular mass markers and nucleosomal histones, respectively. (C) The purified tetrasome was analyzed by 6% non-denaturing polyacrylamide gel electrophoresis with ethidium bromide staining. Lanes 1 and 2 indicate the nucleosome and the tetrasome, respectively. (D) The tetrasome was analyzed by 18% SDS–polyacrylamide gel electrophoresis with Coomassie Brilliant Blue staining. Lanes 1 and 2 indicate histones in the nucleosome and the tetrasome, respectively.

Previously, we successfully evaluated the stabilities of nucleosomes lacking each histone, H2A, H2B, H3, and the H4 N-terminal tail, using SYPRO Orange as a fluorescent dye [30]. This assay allows us to directly detect the histone disassembly from the nucleosomes. In the present study, we performed thermal stability assays with various nucleosomes, the sub-nucleosomal tetrasome, and the histone octamer, and now describe the detailed method for the nucleosome thermal stability assay.

2. Materials and methods

2.1. Preparation of nucleosomes for thermal stability assay

Human histones were bacterially produced by the method described previously [31]. For the nucleosome reconstitution, the 146 base-pair DNA, a palindromic α-satellite DNA derivative, was used [2,32]. The nucleosomes were reconstituted by the salt dialysis method with recombinant human histones H2A, H2B, H3.1, and H4, as described below.

Purified histones H2A (1.2 mg), H2B (1.2 mg), H3.1 (1.4 mg), and H4 (1.0 mg) were mixed in 20 mM Tris–HCl buffer (pH 7.5), containing 20 mM 2-mercaptoethanol and 7 M guanidine hydrochloride, and the histone octamers were reconstituted during dialysis against 20 mM Tris–HCl buffer (pH 7.5), containing 1 mM EDTA, 5 mM 2-mercaptoethanol, and 2 M NaCl. The resulting histone octamers were purified by HiLoad 16/60 Superdex 200 gel filtration chromatography (GE Healthcare). The nucleosomes were then reconstituted by a salt dialysis method with the histone octamer (1.6 mg) and the 146 base-pair DNA (1.0 mg), according to the method previously described [33,34].

Since human H3T does not form a stable histone octamer without DNA [34], the H3T nucleosome was reconstituted with the H2A–H2B and H3T–H4 complexes. H2A (1.2 mg) and H2B (1.2 mg), and H3T (1.4 mg) and H4 (1.0 mg), were separately mixed in 20 mM Tris–HCl buffer (pH 7.5), containing 20 mM dithiothreitol and 7 M guanidine hydrochloride, and the H2A–H2B dimer and the H3T–H4 tetramer were reconstituted during dialysis against 20 mM Tris–HCl buffer (pH 7.5), containing 1 mM EDTA, 5 mM dithiothreitol, and 2 M NaCl. The NaCl concentration of the dialysis buffer was decreased to 100 mM. The resulting H2A–H2B and H3T–H4 complexes were purified by HiLoad 16/60 Superdex 200 gel filtration chromatography. The H3T nucleosome was reconstituted by the salt dialysis method with H2A–H2B (1.0 mg), H3T–H4 (1.0 mg), and the 146 base-pair DNA (1.0 mg).

The H2A.B nucleosome was also reconstituted with the H2A.B–H2B and H3.1–H4 complexes. The H2A.B–H2B dimer and the H3.1–H4 tetramer were prepared by the same method as described previously [35]. The H2A.B nucleosome was reconstituted by the salt dialysis method with H2A.B–H2B (1.0 mg), H3.1–H4 (1.0 mg), and the 146 base-pair DNA (1.0 mg).

For the tetrasome reconstitution, H3.1 (1.4 mg) and H4 (1.0 mg) were mixed in 20 mM Tris–HCl buffer (pH 7.5), containing 20 mM 2-mercaptoethanol and 7 M guanidine hydrochloride, and the histone tetramer was reconstituted during dialysis against 20 mM Tris–HCl buffer (pH 7.5), containing 1 mM EDTA, 5 mM 2-mercaptoethanol, and 2 M NaCl. The resulting H3.1–H4 complex was purified by HiLoad 16/60 Superdex 200 gel filtration chromatography. The tetrasome was reconstituted by the salt dialysis method with H3.1–H4 (0.8 mg) and the 146 base-pair DNA (1.0 mg).

The reconstituted nucleosome and tetrasome samples were subjected to native polyacrylamide gel electrophoresis using a Prep Cell apparatus (Bio-Rad), and the excess free histones and free DNA were removed. The purified nucleosomes and tetrasomes were concentrated, and their concentrations were adjusted to 5 μM with 20 mM Tris–HCl buffer (pH 7.5), containing 1 mM dithiothreitol.

2.2. Thermal stability assay for nucleosomes

The standard reaction conditions are described below. The nucleosomes (2.25 μM) or the tetrasome (2.25 μM) were incubated in the presence of SYPRO Orange for 1 min at 25 °C, in 20 mM Tris–HCl buffer (pH 7.5), containing 1 mM dithiothreitol and 0–200 mM NaCl. In the present study, we used the SYPRO Orange provided by