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Analysis of histones and chromatin in *Xenopus laevis* egg and oocyte extracts

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ABSTRACT

Histones are the major protein components of chromatin, the physiological form of the genome in all eukaryotic cells. Chromatin is the substrate of information-directed biological processes, such as gene regulation and transcription, replication, and mitosis. A long-standing experimental model system to study many of these processes is the extract made from the eggs of the anuran *Xenopus laevis.* Since work in recent years has solidified the importance of post-translational modification of histones in directing biological processes, the study of histones in a biochemically dissectible model such as *Xenopus* is crucial for the understanding of their biological significance. Here we present a rationale and methods for isolating and studying histones and chromatin in different *Xenopus* egg and oocyte extracts. In particular, we present protocols for the preparation of: cell-free egg and oocyte extract; nucleoplasmic extract ("NPE"); biochemical purification of maternally-deposited, stored histones in the oocyte and the egg; assembly of pronuclei in egg extract and the isolation of pronuclear chromatin and histones; and an extract chromatin assembly assay. We also demonstrate aspects of the variability of the system to be mindful of when working with extract and the importance of proper laboratory temperature in preparing quality extracts. We expect that these methods will be of use in promoting further understanding of embryonic chromatin in a unique experimental system.

1. Introduction

Chromatin, a macromolecule composed primarily of DNA and protein, is the physiological form of the genome and ultimately the heritable component of eukaryotic cells. Chromatin contains a basic repeating unit called the nucleosome, composed of DNA wrapped around a core octamer of two each of histones H2A, H2B, H3, and H4 [1]. Chromatin is further condensed and utilized by addition of other proteins, including the linker histone H1 [2], *trans*-acting molecules such as the transcriptional machinery [3], and condensins and cohesins [4]. The histone proteins are highly basic molecules and are frequently modified by a large array of post-translational modifications (PTMs), including: lysine and arginine methylation; lysine acetylation, ubiquitylation, SUMOylation, and ADP-ribosylation; serine, threonine, and tyrosine phosphorylation; and citrullination of methyl-arginine residues [5]. Much evidence now exists to support the hypothesis that the combination of modifications on chromatin-bound histones and the presence and absence of various histone variants contribute to and potentially direct DNA-templated cellular events [6–9]. Therefore the study of chromatin, histones, and their post-translational modifications has become increasingly significant in current research into epigenomics, development, cell-fate, and reprogramming.

While many model systems have been used for the study of chromatin and histones, the vast majority of current work has been conducted in yeast and mammalian cultured cells. Other experimental model systems provide unique opportunities to explore both basic biological phenomena as well as special events at particular life-stages and organism-specific mechanisms. In this piece we focus our attention on a long-standing experimental model whose contributions to our current understanding of fundamental mechanisms underlying the cell cycle are well known, particularly from a biochemical perspective.

The frog *Xenopus laevis* is a well-established laboratory organism, in part because its macroscopic oocytes and eggs are easily obtainable in large number. These oocytes and eggs can be used for many studies in a variety of approaches, including developmental studies and also biochemical studies in cell-free extracts. Much of the seminal work in understanding biochemical aspects of chromatin and histones was performed in cell-free *Xenopus* oocyte and egg extracts [10]. However, in recent years fewer research groups have utilized these remarkable extract preparations, in part due to the inability to genetically manipulate the frogs. In our view, this is a minor impediment as the unique capacity to biochemically

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probe the cell-free extracts and the capacity to utilize defined DNA templates, recombinant proteins, and even the application of heterologous nuclei from cultured cells opens distinct avenues of investigation that are not possible in other models.

Here we present a number of procedures that we utilize to study a variety of aspects of chromatin and histone biology in the cell-free extracts of *Xenopus* oocytes and eggs. These procedures include: brief overviews of the preparation of oocyte, egg, and nucleoplasmic extracts (previously published elsewhere) [11–14]; biochemical purification of maternally-deposited, chaperone-bound stored histones in the oocyte and egg extracts; assembly of pronuclei in egg extract and the isolation of chromatin and its constituent histones; and finally a chromatin assembly activity assay. We highlight some previously unreported caveats to the use of *Xenopus* in the laboratory, including the critical importance of maintaining a low ambient laboratory temperature, and the batch-to-batch variability of extract chromatin assembly activity.

We do note that during the centrifugation of *Xenopus* eggs and oocytes for preparation of extract, maternal chromatin is pelleted and rendered largely unrecoverable amid debris and insoluble material. Regardless, a single maternal chromatin complement would provide a negligible amount of material to study. Therefore, we do not currently have an approach to study the maternally-deposited epigenome. Despite this limitation, we present procedures for the isolation of pronuclei chromatin and histones from assembled sperm nuclei in egg extract; post-S phase, this mimics an early embryo, pre-mid-blastula transition nucleus.

2. Preparation of extracts

Xenopus laevis extracts are prepared from the oocytes or unfertilized eggs of the female *Xenopus* frog. Females are primed for egg-laying with injection of pregnant mare serum gonadotropin (PMSG) to induce oocyte maturation, followed by an injection of human chorionic gonadotropin (HCG) to induce the laying of eggs. To date, four egg extracts have been developed and used for the study of a variety of DNA-templated processes, three of which we will discuss here. The first type is a low-speed interphase supernatant (LSS) [15,16]. This extract has the ability to replicate sperm chromatin through the formation of a transport-competent nucleus. The membrane-free high-speed interphase supernatant (HSS), derived from further clarification of LSS, is replication-incompetent [17], but contains the necessary factors for assembly of the pre-replication complex [18,19] and has the ability to chromatinize small plasmid DNA. The final egg extract we will discuss is the nucleoplasmic extract (NPE) [20], which is obtained by replicating sperm chromatin in LSS. The resulting nuclei are harvested and crushed by centrifugation to yield a highly concentrated, membrane-free nuclear extract. The addition of this extract to either plasmid or sperm chromatin pre-incubated in HSS results in one semi-conservative round of DNA replication.

In addition to the interphase extracts described above, mitotic extracts (trapped in metaphase II of meiosis) can also be prepared from mature eggs in the presence of EGTA [21,22]. EGTA serves to chelate the calcium stores released during egg crushing, preventing activation of the extract into S-phase. The addition of calcium leads to degradation of Cyclin B1 and induces this cytostatic-factor (CSF) arrested extract to undergo a complete round of cell cycle, including mitosis. When prepared in the absence of cycloheximide, this extract can reliably execute multiple rounds of cell cycle. While not discussed herein, this extract might allow careful study of changes in chromatin modification state affected during the cell cycle. For protocols for CSF extract preparation, please refer to Murray [23].

Here, we discuss preparation of extracts from both oocytes and laid eggs [24]. While LSS extracts from both oocytes and eggs are able to assemble chromatin, this activity in egg extracts is typically more robust, perhaps due to the maturation of chaperones required for assembly [25]. Unlike egg extracts, extracts prepared from oocytes are incapable of assembling nuclei or initiating replicative synthesis [26], and therefore are unsuitable for the study of chromatin dynamics during DNA replication. However, oocyte extracts display the distinct advantage of being transcriptionally active, evidenced by high levels of transcription from lampbrush chromosomes during oogenesis [27], allowing for the interrogation of chromatin states during gene activation and transcription. In addition, egg extracts have been instrumental in understanding the biochemical nature of the DNA damage signal [28], and can be further used to investigate changes in chromatin structure upon DNA damage and repair [29,30]. The choice of extract preparation for a particular experiment will depend upon the biological activity that is under investigation. This choice highlights a key feature of the *Xenopus* cell-free systems in that discrete biological events are dissectible by simply modifying the preparation procedure, allowing for precise biochemical studies in a biological context.

Note that for all extract preparations, beginning with egg laying, we find temperature to be a critical aspect of the procedure. We find that the best quality extracts are prepared from eggs laid at 16 °C. In addition, we find that all egg manipulations are best performed at an ambient laboratory temperature of 22 °C or below, preferably 18 °C, in order to have predictable and reproducible extract activities. This point is especially important for the preparation of nucleoplasmic extract. If this is not possible, we recommend bringing all buffers to final volume with 20% cold dH_2O immediately before use.

All animal use and handling should be done in accordance with an IAUCAC-approved protocol. For protocols regarding frog husbandry [31], handling, and injections, refer to the *Xenopus laevis* volume of Methods in Cell Biology [32]. For additional protocols regarding the preparation of egg extracts, please refer to Smythe and Newport [11], Tutter and Walter [12], and Lupardus et al. [13].

2.1. Oocyte extract

2.1.1. Materials and reagents

- Pregnant Mare Serum Gonadotropin (PMSG), Calbiochem #367222
- MS-222, Sigma E10521
- Dissection tools
- 1× Merriam's Buffer: 10 mM Hepes–KOH pH 7.8, 88 mM NaCl, 3.3 mM $Ca(NO_3)_2$, 1 mM KCl, 0.41 mM $CaCl_2$, and 0.82 mM $MgSO_4$
- Dispase II, Roche 04942078001
- Collagenase Type 1A, Sigma C9891
- Sucrose
- Dithiothreitol (DTT, 1 M stock in dH_2O, store at −20 °C)
- 13 × 51 polycarbonate tubes, Beckman #349622
- Ultracentrifuge (Beckman SW-55 rotor)

2.1.2. Procedure for preparation of oocyte extract

Select 1–4 adult female *Xenopus* frogs that have not laid eggs within the prior 3 months and prime them with 50 U PMSG 3–5 days prior to use in order to maximize the quantity of matured, late stage oocytes. Anesthetize the frogs with MS-222 and sacrifice them according to standard and IAUCAC-approved protocols. Immediately dissect out the entire ovaries, which are found in the abdomen, to a beaker. The ovaries will be easily removed intact, and should contain a large number of obviously mature and large oocytes with clearly pigmented and marked layers; mature stage VI oocytes look almost identical to laid eggs. First, grossly disrupt the ovaries using dissection scissors. Next, disrupt the follicular cell layer with a 3 h digestion with the mild protease