Topic Introduction

Reconstituting Nuclear and Chromosome Dynamics Using *Xenopus* Extracts

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Extracts prepared from the eggs of frogs, particularly *Xenopus* species, have provided critical material for seminal studies of nuclear and chromosome dynamics over several decades. Their usefulness for these types of analyses lies in several important characteristics: stockpiled nuclear components, absence of endogenous DNA, and intact and functioning signaling networks. These factors have allowed detailed molecular analyses of many aspects of chromosome biology, including DNA replication, checkpoint signaling, epigenetic control, and chromosome condensation, cohesion, and segregation. In this introduction, the preparation and application of *Xenopus* egg extracts for the study of chromosomes and chromatin are described in detail.

BACKGROUND

Early nuclear transfer experiments suggested that functional changes were exerted on somatic nuclei when they were incubated in the cytoplasm of a *Xenopus* egg, and that these changes represented reversal of cellular differentiation, restoring the nuclei to pluripotency (Gurdon et al. 1958; Gurdon 1962a,b). These studies eventually led to the notion that embryonic extracts might also be exploited to understand other aspects of nuclear function. In the early 1980s, Lohka and Masui showed that demembranated sperm nuclei added to extracts prepared from frog eggs, in this case *Rana pipiens*, spontaneously underwent the nuclear events that had previously been reported to occur in embryos following fertilization of the egg (Lohka and Masui 1983). These included decondensation of the sperm chromatin, nuclear envelope assembly, and DNA replication. The fact that these events occurred spontaneously in the extracts supported the notion that this system could be exploited to understand their molecular underpinnings. Murray and Kirschner (Murray et al. 1989; Murray and Kirschner 1989) showed that extracts could be prepared from *Xenopus* eggs that spontaneously cycled between the different phases of the cell cycle, and that this cycling was accompanied by and dependent on fluctuations in the level of a protein “cyclin,” an essential component of the previously described mitosis promoting factor, or MPF (Gerhart et al. 1984; Minshull et al. 1989). This system has since been exploited for many groundbreaking investigations of the fundamental mechanisms of nuclear and chromosome dynamics. Drugs, proteins, nucleotides, and DNA templates can be added to the extracts with ease. In addition, proteins can be depleted from extracts using appropriate antibodies,

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From the *Xenopus* collection, edited by Hazel L. Sive.

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Cite this introduction as *Cold Spring Harb Protoc*; doi:10.1101/pdb.top097105

allowing analysis of mutant recombinant proteins in rescue experiments. This ability is particularly useful if the proteins under investigation are essential for viability, which can make their study in somatic cells technically challenging.

MANIPULATION OF CELL CYCLE STATE IN VITRO

The ability to manipulate the cell cycle state makes *Xenopus* egg extracts a powerful tool for the in vitro study of chromatin and chromosome dynamics. Mature *Xenopus* eggs are arrested in metaphase of the second meiotic division (MII) with high levels of meiotic kinase activity. This arrest, mediated by cytosolic factor (CSF), is relatively stable. Extract prepared directly from eggs (“CSF extract”) can be exploited to study the impact of M phase activities on chromosome and chromatin behavior and events (Fig. 1). Additionally, because CSF arrest can be released by the addition of calcium to the extract, CSF extract can also be used to study the impact of mitotic exit on chromosome dynamics. Extracts induced to enter interphase by the addition of calcium can be maintained in interphase by preventing translation of endogenous cyclin RNA.

CSF activity is dominant and can be used to drive interphase extracts into M phase. Freshly prepared or thawed frozen CSF extract added in equal volume to interphase extract causes the entire mixture to enter M phase, where it will again arrest. This kind of approach is particularly useful if events to be analyzed in M phase, such as sister chromatid cohesion, are dependent on prior DNA replication.

NUCLEI, CHROMOSOMES, AND CHROMATIN IN VITRO

A variety of DNA substrates can be added to egg extract to assess different aspects of chromatin function and regulation, chromosome dynamics, and nuclear assembly (Fig. 1).

Sperm nuclei can be isolated from *Xenopus* testes and demembrated with a mild detergent extraction, as described in Protocol: **Isolation and Demembration of *Xenopus* Sperm Nuclei** (Hazel and Gatlin 2018). This method of sperm preparation enables reconstitution of chromosome reactions in vitro and allows the simultaneous addition of many nuclei to the extract. Nuclear assembly occurs spontaneously when demembrated sperm nuclei are added to interphase extract and can be analyzed as described in Protocol: **Nucleus Assembly and Import in *Xenopus laevis* Egg Extract** (Chen and Levy 2018). This approach was critical to the demonstration that nuclear envelope assembly is promoted by chromatin-dependent remodeling of the endoplasmic reticulum (Anderson and Hetzer 2007), and has enabled seminal findings about the biochemistry of nuclear import (Görlich et al. 1994). Egg extract was also used to provide insight into the mechanisms of nuclear disassembly in M phase (Mühlhäusser and Kutay 2007).

Early work from several labs established *Xenopus* egg extract as a uniquely powerful system in which to study DNA replication and its regulation (Blow and Laskey 1986; Blow and Watson 1987; Blow 1993; Walter and Newport 1997, 2000). Complete DNA replication occurs spontaneously in <2 h when sperm nuclei are added to crude interphase egg extract through the recruitment of proteins and nucleotides from the extract. The roles of particular replication proteins can be dissected by immunodepletion, and recombinant and mutant derivatives can be used to rescue function. This system has been used to elucidate how replication licensing is entrained to cell cycle progression (McGarry and Kirschner 1998; Wohlschlegel et al. 2000), and to define the mechanism by which DNA replication promotes degradation of certain replication factors (Havens and Walter 2009), as well as many other aspects of DNA replication and repair.

The in vitro study of DNA replication was further refined by Walter, Newport, and colleagues (Walter et al. 1998), who established a method for nucleus-free DNA replication. Whereas the critical early replication-licensing step occurs in clarified high-speed extract, replication initiation requires

FIGURE 1. The use of *Xenopus* egg extracts to study chromatin and chromosome dynamics. CSF extracts, which contain high levels of MPF (mitosis promoting factor) activity are prepared directly from *Xenopus* eggs, which are arrested in M phase of meiosis II. CSF extracts can be used to study events that require M phase activity, such as centromere and kinetochore assembly, mitotic checkpoint function, and chromosome condensation (shown at *top*). The addition of Ca^{++} releases CSF arrest, resulting in cyclin degradation, and entry into interphase (*middle*). Interphase extract can be used to study events such as DNA replication and nuclear assembly and import (shown at *bottom*). Interphase extract, which contains abundant membrane stores, can also be further fractionated by centrifugation to study licensed DNA replication in a nucleus-free system. This requires the preparation of two different extracts, one enriched in nuclear activities (nucleoplasmic extract or NPE), and the other a membrane-free supernatant, as shown at *right*. Some studies, such as chromosome cohesion assays, require cycling of the extract through interphase and back into M phase. This is achieved by adding CSF extract to the reaction following interphase incubation. CSF promotes M phase entry and arrest in a dominant manner, resulting in condensation of replicated chromosomes. Some assays require multiple cell cycles *in vitro*, which can be achieved by preparation of cycling extract (*center*). Finally, developmental changes in chromosome and chromatin regulation can be studied by preparation of developmentally staged embryonic extracts or nuclei (*bottom left*). These are prepared from embryos collected at the desired developmental stage after fertilization. The manipulations shown in this schematic can also be applied to other questions of vertebrate chromosome and chromatin regulation as new questions arise.

nuclear assembly. This requirement for nuclear assembly can be bypassed by sequential incubation of DNA substrates in clarified interphase extract to promote replication licensing, followed by incubation in concentrated nucleoplasmic extract (NPE). NPE is prepared in advance from nuclei assembled in crude interphase extract and stored frozen in aliquots. This powerful system is described in detail in Protocol: **Extracts for Analysis of DNA Replication in a Nucleus-Free System** (Sparks and Walter 2018). Importantly, this system allows the complete replication of plasmids and other small templates that are not efficiently replicated in interphase extract, and has been exploited to define intermediates in replication initiation and related events (Walter 2000; Takahashi et al. 2004).

During nuclear assembly in interphase extract, histones are recruited from the extract and nucleosomes are assembled on the DNA. The complement of histone variants and modifications deployed in different cell types is developmentally regulated. Extracts prepared from eggs and embryos

at different developmental stages can be used to study these processes in detail. This approach is described in Protocol: **Chromatin Characterization in *Xenopus laevis* Cell-Free Egg Extracts and Embryos** (Wang et al. 2018). Using this method, it has been possible to correlate certain histone modification signatures with differentiation status (Shechter et al. 2009).

Egg extracts can also be used to study specialized forms of chromatin, such as centromeres, and how they are formed. Methods to tackle these questions are described in Protocol: **Centromere and Kinetochore Assembly in *Xenopus laevis* Egg Extract** (Flores Servin and Straight 2018), which includes descriptions of how to study kinetochore and centromere assembly on intact sperm chromosomes, as well as methods to prepare beads coated with model DNA substrates. Using immunodepletion and rescue, in combination with mutant protein analysis in these assays, the dependencies and functional interactions of various centromere- and kinetochore-associated factors can be assessed. The readouts can be both biochemical and microscopy based. This system has been used to show that sequences in the histone variant CENP-A that promote histone assembly are distinct from those that ensure centromere function (Guse et al. 2011).

The morphology of in vitro assembled whole condensed chromosomes can be assessed using egg extracts, as described in Protocol: **Chromosome Cohesion and Condensation in *Xenopus* Egg Extracts** (da Silva and Rankin 2018). The chromosomes of sperm nuclei added directly to CSF extracts are chromatinized and condensed to form single chromatids, all through the activity of extract-associated proteins. This approach allowed the earliest biochemical identification of chromosome condensation factors (Hirano and Mitchison 1994). The ability to cycle extracts through interphase, in which DNA replication occurs, and back into M phase also allows exploration of mechanisms that control cohesion between sister chromatids, and has been very useful for studying the relationship between DNA replication and cohesion establishment (Losada et al. 1998; Sumara et al. 2000; Gillespie and Hirano 2004; Takahashi et al. 2004; Rankin et al. 2005). Importantly, because many cohesion and condensation factors are essential, the use a depletion and rescue approach in egg extracts bypasses the complication this presents when working in somatic cells. Rapid and synchronous genome duplication in egg extracts make these studies practical.

Xenopus egg extracts can also be used to study signaling events that depend on chromatin or nuclear activity. For example, the mitotic checkpoint, or “spindle checkpoint” is a mechanism that prevents progression out of M phase in the presence of unattached kinetochores. Unattached kinetochores generate a soluble signal, which ultimately prevents cyclin degradation, resulting in sustained mitotic arrest. The mitotic checkpoint can be artificially generated in egg extract by adding a high concentration of sperm nuclei together with a microtubule depolymerizing drug, as described in Protocol: **Analysis of Mitotic Checkpoint Function in *Xenopus* Egg Extracts** (Mao 2018). In this system, mitotic arrest is monitored by assaying for mitotic kinase activity, or by assessing nuclear morphology. This method can be used to assess kinetochore function, as well as to dissect the mitotic checkpoint mechanism itself (Minshull et al. 1994). Other chromatin-dependent signaling events, such as the Ran GTPase gradient (Kalab et al. 1999; Zhang and Clarke 2000; Wiese et al. 2001), and DNA damage signaling (Lupardus et al. 2007; Costanzo et al. 2004) have also been successfully characterized using the egg extract system. This flexible system can be further adapted and applied to new questions as they arise.

SUMMARY

Since the discovery that the events of cell division and their regulation could be recapitulated in vitro using extract prepared from *Xenopus* eggs, a wide variety of chromatin and chromosome-based events and mechanisms have been elucidated using this powerful system. The protocols discussed herein provide an introduction to the use of extracts to study questions of chromatin, chromosomes, and signaling events that control their activity.

ACKNOWLEDGMENTS

I am grateful to all members of the Rankin laboratory for their continued support and enthusiasm, Romain Gibeaux for components of the illustration, and to all investigators, past and present, who helped develop the mighty *Xenopus* extract system. This work was supported by National Institutes of Health (NIH) R01 GM101250, and a grant from the Oklahoma Center for Adult Stem Cell Research, to S.R.

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Cold Spring Harb Protoc; doi: 10.1101/pdb.top097105 originally published online August 27, 2018

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