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Analyzing the ATR-mediated checkpoint using *Xenopus* egg extracts

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Abstract

Our knowledge of cell cycle events such as DNA replication and mitosis has been advanced significantly through the use of *Xenopus* egg extracts as a model system. More recently, *Xenopus* extracts have been used to investigate the cellular mechanisms that ensure accurate and complete duplication of the genome, processes otherwise known as the DNA damage and replication checkpoints. Here we describe several *Xenopus* extract methods that have advanced the study of the ATR-mediated checkpoints. These include a protocol for the preparation of nucleoplasmic extract (NPE), which is a soluble extract system useful for studying nuclear events such as DNA replication and checkpoints. In addition, we describe several key assays for studying checkpoint activation as well as methods for using small DNA structures to activate ATR.

Keywords: ATR; Chk1; Rad1; Hus1; Rad9; Checkpoint; *Xenopus*; NPE; Chromatin; Chloroquine; Replication

1. Introduction

The maintenance of genomic integrity is essential for the survival of all organisms. As a result, cells have evolved multiple processes to prevent genomic instability caused by normal DNA metabolism and insults from DNA damaging agents or replication inhibitors. These processes, which are coordinated by the DNA damage and replication checkpoints, include arrest of cell cycle progression, initiation of apoptosis, regulation of DNA repair, and maintenance of replication fork stability [1,2]. In eukaryotes, checkpoint activation is centrally mediated by the ATR (Ataxia Telangiectasia and Rad3-related) kinase and its homologs [3], which in combination with a number of other checkpoint proteins forms a signaling complex competent to phosphorylate and activate downstream targets [4].

The ATR-mediated checkpoint pathways are highly conserved, and similar mechanisms of checkpoint activation are found in organisms as divergent from humans as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [4]. The evolutionarily conserved nature of this response has permitted the use of a number of systems for checkpoint analysis, ranging from yeast genetic systems to transgenic mice. These systems have provided valuable insights into the processes that lead to checkpoint activation, as have biochemical studies with purified proteins. Yet as activation of the checkpoint following many types of DNA damage has been linked to DNA replication [5–8], the *Xenopus* egg extract system has offered several advantages for analysis of checkpoint signaling and activation. This *in vitro* system involves the use of extracts prepared from the eggs of *Xenopus laevis* which can cycle through S and M phase in a highly synchronous manner, replicating chromatin in a semi-conservative fashion [9–13].

Indeed, various types of *Xenopus* egg extracts have proven to be quite effective for the study of DNA replication and these studies have revealed that replication in extracts occurs through mechanisms very similar to those in mammalian cells [reviewed in [14]]. The cell-free nature of the system allows extensive manipulation of the proteins present, through immunodepletion and protein addition experiments, as well as alteration of the DNA template,

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which can take the form of chromatin, plasmid or even simpler structures. For the cell cycle checkpoint field, this has allowed a level of biochemical analysis difficult to achieve in mammalian cells due to the essential nature of many proteins involved in checkpoint activation. Furthermore, these extracts have allowed the assessment of biochemical events reconstituted with purified proteins in a system that more closely resembles that of a cell.

Here we describe recently developed methods that are useful for the study of checkpoint activation in *Xenopus* extracts. Specifically, we outline the preparation of nucleoplasmic extracts (NPE), which is a relatively new and powerful addition to the repertoire of *Xenopus* extracts [15]. These extracts have the ability to replicate double-stranded plasmid templates [15,16], and have proven useful for understanding the connection between DNA replication and the nature of the DNA damage signal [17]. Additionally, we discuss our experience using ATR-mediated phosphorylation events for studying activation of the checkpoint. Lastly, we discuss the use of plasmid-based DNA templates for activating ATR, as well as methods for assessing the replication of these templates in *Xenopus* extracts.

2. Extract preparation

Four different types of extracts derived from *Xenopus* eggs have been used for checkpoint studies. The first type is a low-speed (interphase) extract (LSE). To prepare this type of extract, eggs are harvested and centrifuged to separate the yolk, pigment, and lipid contents from the cytoplasm. The result is a cytoplasmic extract containing small amounts of lipids that is competent for nuclear formation and replication of chromatin [9,10], and arrests in interphase following completion of replication. A second type of low speed extract, called cytostatic-factor arrested (CSF) extract, can be used to recapitulate a full cell cycle, including mitosis [18,19]. High speed centrifugation of LSE to remove membrane components produces the third type of extract, a high-speed extract (HSE) consisting of egg cytosol devoid of all lipid content [20,21]. HSE is capable of single-stranded M13 replication [21] and pre-RC formation [16]; however, it cannot promote firing of origins of replication. The fourth type of extract is a nucleoplasmic extract (NPE), which is obtained by replicating sperm chromatin in LSE [15]. The nuclei that form upon incubation of sperm chromatin with LSE are isolated and centrifuged to yield the highly concentrated, membrane-free NPE. Both chromatin and double-stranded plasmids can be replicated by sequential incubation in HSE followed by NPE [16].

2.1. Preparation of Xenopus interphase (LSE) and cytosolic (HSE) extracts

Xenopus cell-free extracts are obtained from the unfertilized eggs of female *X. laevis* frogs. To induce egg production, female frogs are "primed" to lay eggs by injection of pregnant mare serum gonadotropin (PMSG), followed by an injection of human chorionic gonadotropin (HCG) several days later to induce the laying of eggs. Harvested eggs are then crushed by centrifugation and the cytoplasmic layer is isolated for use. For in-depth information on frog injection and egg collection techniques, we direct the reader to the excellent protocols previously described by Murray [22], Walter and Newport [23] and Tutter and Walter [24]. These papers are also excellent references for the preparation of LSE, HSE, NPE and CSF extract, as well as the preparation of demembranated sperm chromatin from male *X. laevis* frogs.

2.1.1. Special considerations for the preparation of HSE

When making LSE in preparation for HSE, care should be taken to remove only the light brown cytoplasmic layer while completely avoiding both the yolk and pigment below and the lipids at the top. We have also found that gently inverting the LSE 60–80 times prior to centrifugation increases the yield of HSE following ultracentrifugation.

2.2. Preparation of Xenopus nucleoplasmic extract (NPE)

First developed by Walter et al. [15], NPE is a highly concentrated nuclear fraction that can be used in combination with HSE to replicate both chromatin and double-stranded plasmids.

2.2.1. Special considerations for the preparation of NPE

2.2.1.1. LSE. When making LSE in preparation for NPE, it is helpful to completely remove the middle cytoplasmic layer until the lipid and pigment layers touch. During this process, invariably a small amount of the yellow lipid layer is also removed with the cytoplasmic layer. We find that these extra lipids removed with the cytoplasmic extract aid in nuclear formation following addition of chromatin.

2.2.1.2. Sperm chromatin. Optimal nuclei formation occurs around 4000 nuclei/μL of extract. Typically, this is achieved by adding the appropriate amount of a 250,000 nuclei/μL chromatin stock to the extract. Unfortunately, the percentage of viable sperm heads (i.e., sperm that can form good nuclei) varies between preparations. To account for this variability, after a new chromatin preparation is made, a number of different dilutions of sperm (for example, 3000, 4000, and 5000 nuclei/μL) are tested to find the optimal concentration at which the largest nuclear layer is obtained. Each dilution of new sperm chromatin is tested in a 4.5 mL batch against a previously characterized, control sperm preparation at its optimal dilution. Note that if the volume of sperm to be added is under 100 μL, the sperm should be diluted 1:1 with sperm dilution buffer prior to addition to the LSE.

2.2.1.3. Nuclear layer. When pipetting the nuclear layer, the viscosity should be such that the nuclei are somewhat