Final Project

BIOL 463 – Gene Regulation in Development

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Is DNA Polymerase α, a component of the eukaryotic replisome, necessary for the deposition of H2A and H2B onto nascent DNA in *Saccharomyces cerevisiae*?

Background Information:

The replisome is a complex of proteins in eukaryotic cells that carries out the replication of genetic material. It consists of many different proteins, only a few of which are of interest to this paper and will thus be discussed in this section. DNA polymerase α (Pol α) is a component of the replisome in eukaryotic cells that initiates the formation of Okazaki fragments during lagging strand synthesis. The FACT complex (FAcilitates Chromatin Transcription) allows transcription of the DNA, and is believed to help stabilize the nucleosomal structure throughout the process of replication (Winkler and Luger 2011). CMG Helicase assists in the unwinding of the DNA to allow replication proteins to continue through the replication fork. The amino terminus of Pol α (Pol1) binds to the mcm2 subunit of CMG Helicase and FACT, connecting them in the replisome. Additionally, FACT and the mcm2 subunit have both been shown to bind Histone proteins H3 and H4 (Huang et al. 2015, Martin 2018)

A recent study has shown that the Pol1 region of Pol α also contains a highly-conserved binding motif that is specific for Histone proteins H2A and H2B, and is also necessary for maintaining gene-silencing in telomeric and mating-type loci following replication (Evrin et al. 2018.) This was shown by observing colonies of *Saccharomyces cerevisiae* with color-changing reporter constructs that were WT for the histone-binding ability of Pol1, and in cells that were mutants for the histone-binding ability (and thus could no longer bind histones). It was further shown in this study that disrupting the histone-binding motif is not necessary for propagating DNA synthesis. One inference that can be made from this is that the histone-binding abilities of Pol1 are used at some point after the replication of DNA, as DNA synthesis still proceeds regardless of Pol1's ability to bind histone proteins.

It is still unclear how this process works to maintain gene-silencing following replication. As Pol α is bound to the mcm2 subunit and FACT, and one possible inference is that Pol1 binds histones after replication, it is possible that these three proteins assist in the formation of nucleosomes following transcription by assisting in the deposition of histone complexes. The purpose of this experiment is to explore whether Pol α is necessary for the proper formation of nucleosomes following replication. We will focus on Pol α as it will be easier to manage only one

variable at a time rather than examining three proteins at the same time, and since the proper functioning of Pol α was shown to be necessary to maintain gene-silencing following replication.

Relevance and Impact:

Although some histone chaperones have been shown to deposit histones onto newly-replicated DNA (Park and Luger 2008), there is still much that is unknown about this complicated process. It is possible that the DNA replication machinery (e.g. Pol α) plays a dual role in stripping histones and proteins off the DNA during replication, and replacing them immediately after. There is little research done in this field, although it is an important process to understand.

Understanding of the functions of the replisome machinery will help pave the way for future studies that investigate the role of chromatin in mediating epigenetic inheritance. Characterizing the proteins responsible for the reassembly of nucleosomes after replication will allow future researchers to study modes of inheritance, and investigate how epigenetic markers, such as histone placement and modifications, are conserved across generations of cells. This experiment should provide a deeper understanding of the functions of the replisome components in *S. cerevisiae*, which will provide a useful foundation for exploring other eukaryotic systems with more complex regulatory systems, such as plants and eventually humans.

In turn, understanding the mechanisms behind epigenetic inheritance and the formation of chromatin architecture may allow future researchers to develop a wide variety of techniques and concrete applications. For example, future studies may investigate how to modify epigenetically silenced genes or prevent these genes from being silenced by modifying the proteins responsible for their silencing. It will also be possible to further understand how epigenetically-regulated phenotypes and diseases are conserved through generations of cells, and provide insight into how expression of these genes may be controlled.

Hypothesis and Predictions:

It has been established that proper binding of the Pol1 subunit of Pol α to histones is not necessary for the proper replication of DNA strands, but it is instead necessary for the proper silencing of genes at telomeric and mating-type loci (Evrin et al. 2018). As such, one logical inference would be that the histone-binding ability of the Pol1 subunit is necessary for a step in the replication process that occurs after the strands have been replicated.

It is also known that the Pol1 subunit of Pol α contains a conserved binding site for histone proteins H2A and H2B, and also binds to FACT and the mcm subunit of CMG helicase (both of which have been shown to bind H3 and H4) (Evrin et al. 2018). Histone complexes were also found to be bound *in vivo* to Pol α, FACT, and CMG helicase (Martin et al. 2018). As these three replisomes proteins are linked together in the replisome and have been found to simultaneously

bind histone complexes, it is possible that the three of them work in concert to deposit histones onto nascent DNA following replication. The proper deposition of histones is required for the formation of nucleosomes, and is thus the foundation of chromatin architecture.

Given these, I hypothesize that Pol α is necessary for the proper formation of nucleosomes following replication in *S. cerevisiae*. I believe that Pol α is responsible for replacing histones onto nascent DNA following replication, and that mutations of Pol α will result in a lack of deposition of histones onto nascent DNA, thus preventing the proper condensation of chromatin. It is also known that linker DNA (DNA not bound by histones) is much more accessible to these enzymatic proteins, and is thus much more susceptible to digestion (Heins et al. 1967). It is important to note that if Pol α is necessary for the proper formation of nucleosomes by assisting in histone deposition, genetic material extracted from cells with improperly condensed chromatin will be more susceptible to digestion by nuclease proteins (Nobile et al. 1986). This increase in digestibility is integral to the experimental procedure of this study.

It is also possible that the histone-binding activity of Pol α is used during transcription to strip histones off of chromatin and allow proteins to read and process through the DNA. However, I believe that this is an unlikely process, as it would not explain the necessity of Pol α for the inheritance of epigenetic markers. As such, this experiment will solely test Pol α's function in replication.

Experimental Procedures:

I. Developing the Cell System

I will use a line of *S. cerevisiae* cells as the system to investigate whether Pol α is necessary for the formation of nucleosomes as their replisome has been well-characterized, and they have been the system of choice for the previous studies that this experiment is founded on. They are a practical choice as they are eukaryotic cells with a short doubling time, and provide a suitable model for more complex eukaryotes that can be studied further down the line.

To study the necessity of Pol α, I will develop two lines of cells: one cell line that is WT for Pol α as the control, and one cell line with a known temperature-sensitive mutation in the Pol 1 subunit as the variant. The WT cell line is an important control as it will set the base level of nucleosomes present in *S. cerevisiae*. A decrease in the amount of nucleosomes present in the variant line can thus be attributed to the mutation of Pol α.

II. DNA Replication & Western Blot

All cells will be arrested in the G1 phase through treatment with alpha factor, a chemical that prevents yeast from proceeding through replication. Both cell lines will be raised to a temperature of 37 degrees Celsius in order to inactivate the heat-sensitive Pol α, and keep the control line in the same conditions as the variant. The cell lines will then be allowed to proceed through S phase synchronously by repeatedly washing off alpha factor. Allowing cells to proceed

through S phase and arresting them at the same point in time will minimize error caused by cells having different amounts of DNA because of asynchronous DNA replication. This assures that the cells have just gone through replication, and that the changes made to chromosome structure can be attributed to DNA replication. Thus, this allows us to clarify that the histone-depositing activity of Pol α occurs during DNA replication (Recall, it may be possible that Pol α functions in histone depositing during transcription instead of replication).

After the cells have had sufficient time to replicate their DNA, they will be arrested in the G2 phase. After the cells are arrested in G2 phase, cells will be cross-linked, lysed, and their genetic material extracted. Cross-linking will ensure that the DNA is bound to histones and remains in the same form that it would have been *in vivo*. Lysing the cells is necessary for extracting their genetic material, which will then be further analyzed.

Samples for western blots will also be drawn from the culture before the cell lines are raised to 37 degrees Celsius and after they are raised to 37 degrees Celsius. This will ensure that the protein is still expressed and present in the cell culture, and its expression has not been affected by the mutation in the Pol 1 subunit.

III. MNase Digestion

MNase (Micrococcal nuclease) is a nuclease commonly used to map nucleosome position on *S. cerevisiae* by digesting DNA that is not bound by histones. DNA that is bound by histones or other regulatory proteins are protected from MNase digestion (Herrmann et al. 2017). Incomplete MNase digestion results in a DNA ladder on an agarose gel where the bands indicate the amount of mononucleosome, dinucleosome, trinucleosome, etc. If the reaction is carried to completion, only the band indicating the mononucleosome would be present on the agarose gel analysis as all the linker DNA would have been digested. A typical MNase digestion is pictured below. If the genetic material is more accessible to the MNase (e.g. if there is a higher concentration of MNase, if the reaction is allowed to proceed longer, if there are less histones etc.), then you would expect less of the higher number of nucleosome fragments, and more mononucleosomes (as the DNA would have been much further digested.)

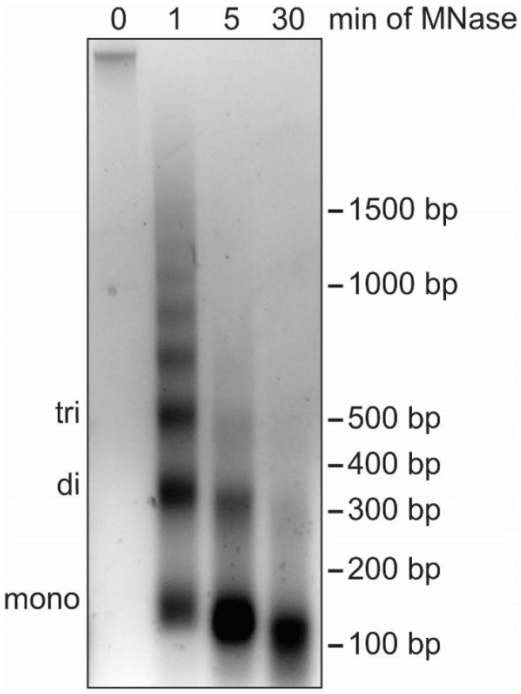

Figure 4 from Herrmann et al. showing an agarose gel analysis of MNase-digested DNA. Undigested DNA (0min MNase digestion in lane 1) shows that the DNA is not degraded by any endogenous enzymes. The position of the bands on the gel in the succeeding lanes correspond to the mono-, di-, tri-, and polynucleosomes (mono-, di-, trinucleosomes labeled on the left axis). Bands are located within intervals of approximately 150bp (the length of one nucleosome.) With increased time of MNase digestion (increased exposure to MNase,) less of the higher-order polynucleosomes are present, and more of the lower-order or mononucleosomes are present.

The genetic material extracted from the *S. cerevisiae* cells will then be subject to MNase digestion. Genetic material from each sample will be digested for different amounts of time (0, 1, 2.5, 5, 10, 20 minutes), and one sample will also be left without any MNase. The MNase digestion will be halted with EDTA, a chelating agent used to halt nuclease activity. The 0-minute sample is an important control to show that the starting genetic material is present and is undigested. The sample without any MNase is a control to show that the genetic material is being digested only by the MNase added as part of the experiment, and is not digested by any endogenous nucleases present in the cell. DNA from both lines will be digested for varying lengths of time to show how quickly the DNA is being digested, giving us an idea of how accessible the DNA is to the MNase (and therefore whether or not the nucleosomes were properly formed). This will also provide more data points to provide further confidence in the findings of the experiment.

Results and Interpretations:

Result #1: There is an increase in DNA digestion in the Pol α mutant when compared to the WT cell line. This would be indicated by a faster disappearance of the heavier bands on the gel, and a higher abundance of the mononucleosome bands at the bottom of the gel.

This is the predicted outcome of the experiment, as it would indicate that the DNA in the Pol α mutant is more susceptible to MNase digestion. As such, we can infer that the DNA in the Pol α mutant is less densely packed than in the WT cell line. Pol α therefore is necessary for the proper formation of nucleosomes following replication. The histone-binding motif of Pol1 would likely be responsible for depositing H2A and H2B onto the nascent DNA, as these are the histone

proteins it contains a binding site for. Since Pol α was also shown to be bound to FACT and the mcm2 subunit of CMG helicase, it is possible that these three proteins would work in concert to deposit the histone octamer onto nascent DNA. This result would agree with the previously mentioned study that found that Pol α, FACT, and the mcm2 subunit are necessary for maintaining gene-silencing following replication (Evrin et al. 2018). The deposition of histone proteins and formation of nucleosomes would be vital to chromatin architecture, and thus for maintaining epigenetic modifications required for gene-silencing.

If these are the results of the experiment, it would be interesting to further examine mcm2 and FACT mutants in a similar experiment to this one to see if these proteins also play a vital role in maintaining chromatin structure. It may also be fruitful to confirm that Pol α is responsible specifically for H2A and H2B placement onto nascent DNA by radiolabelling these proteins and checking for the presence of these radiolabelled histones in POL 1 variant cell lines that have undergone replication.

Result #2: There is no difference in the digestion of DNA from the Pol α mutant line and the WT cell line. This would be indicated by near-identical banding patterns on the gel.

This would indicate that there is no change in the susceptibility of the two cell lines to MNase digestion. As such, we can infer that there is no change to nucleosome formation nor chromosome architecture. Pol α therefore is not necessary for the proper deposition of histones onto nascent DNA. It is possible that there is a redundant protein that would deposit these histones onto the nascent DNA, and are adequate in the absence of Pol α. For example, FACT and mcm2 may also contain binding sites for H2A and H2B that are yet to be discovered. It would be interesting to further examine specific subunits of the FACT complex in this type of experiment to determine whether they are responsible for depositing histones following replication, as FACT is usually bound to destabilized nucleosomes (Martin 2018). It may be possible that FACT assists in the stabilizing of DNA during replication, and also assist in maintaining histone placement when DNA is destabilized.

Another possibility is that Pol α plays no role in the deposition of histones at all. The histone-binding motif on Pol1 may function later on in the DNA maturation process. Since Pol α is necessary for maintaining gene-silencing following replication, it is also possible that Pol α recognizes histones after replication, and recruits methylases or other histone writers that would induce epigenetic silencing after replication.

Result #3: There is a decrease in DNA digestion in the Pol α mutant when compared to the WT cell line. This would be indicated by a greater amount of the heavier bands on the gel, and a lower abundance of the mononucleosome bands at the bottom of the gel.

This would indicate that the DNA in the Pol α mutant is less susceptible to MNase digestion. As such, we can infer that the DNA in the Pol α mutant is more densely packed than in the WT cell line. This would be an unexpected result as Pol α has been shown to be necessary for

maintaining gene-silencing following replication, and a result that suggests that the Pol α mutant results in a more densely packed chromatin would be contradictory to this. One possibility is that WT Pol α recruits methylases to the chromatin after replication, and these methylases were cross-linked to the DNA during the experiment. If a significant enough amount of methylase were cross-linked to the chromatin, it could block the DNA from the MNase, thus making it less susceptible to digestion.

References:

Evrin, Cecile, et al. “Histone H2A-H2B Binding by Pol α in the Eukaryotic Replisome Contributes to the Maintenance of Repressive Chromatin.” The EMBO Journal, vol. 37, no. 19, 2018, doi:10.15252/embj.201899021.

Heins, James N., et al. “Characterization of a Nuclease Produced by Staphylococcus Aureus.” Journal of Biological Chemistry, vol. 242, 10 May 1967, pp. 1016 - 1020., doi:10.1515/znb-1969-0820.

Herrmann, Christin, et al. “Differential Salt Fractionation of Nuclei to Analyze Chromatin-Associated Proteins from Cultured Mammalian Cells.” Bio-Protocol, vol. 7, no. 6, 2017, doi:10.21769/bioprotoc.2175.

Huang, Hongda, et al. “A Unique Binding Mode Enables MCM2 to Chaperone Histones H3–H4 at Replication Forks.” Nature Structural & Molecular Biology, vol. 22, no. 8, 2015, pp. 618–626., doi:10.1038/nsmb.3055.

Kunkel, Thomas A., and Peter M. J. Burgers. “Arranging Eukaryotic Nuclear DNA Polymerases for Replication.” BioEssays, vol. 39, no. 8, 2017, p. 1700070., doi:10.1002/bies.201700070.

Martin, Benjamin J. E., et al. “Transcription Promotes the Interaction of the FAcilitates Chromatin Transactions (FACT) Complex with Nucleosomes InS. Cerevisiae.” 2018, doi:10.1101/376129.

Nobile, C, et al. “Nucleosome Phasing on a DNA Fragment from the Replication Origin of Simian Virus 40 and Rephasing upon Cruciform Formation of the DNA.” Molecular and Cellular Biology, vol. 6, no. 8, 1986, pp. 2916–2922., doi:10.1128/mcb.6.8.2916.

Park, Young-Jun, and Karolin Luger. “Histone Chaperones in Nucleosome Eviction and Histone Exchange.” Current Opinion in Structural Biology, vol. 18, no. 3, 2008, pp. 282–289., doi:10.1016/j.sbi.2008.04.003.

Winkler Duane D., Luger Karolin. “The histone chaperone FACT: structural insights and mechanisms for nucleosome reorganization.” J. Biol. Chem. Vol 286, pp. 18369 - 18374. 2011, doi:10.1074/jbc.R110.180778