The dynamic organization of prokaryotic genomes: DNA bridging and wrapping proteins across the tree of life

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Chapter 7

General discussion

Across the tree of life, organisms have evolved ways of organizing their chromosomes and regulating gene expression. Chromatin is dynamically organized and structured as a function of growth cycle and responds to environmental factors and specific physico-chemical cues. The tree of life consists of two major branches: the bacterial and the archaeal/eukaryotic branch (1). Eukaryotes structure their chromosomes locally using nucleosomes consisting of an octameric protein core with DNA wrapped around it (2). Most flexibility in this structure comes from either post-translational modifications (PTMs) on the histone tails or the inclusion of histone variants (3–6). These variants are often involved in specific processes such as DNA damage repair, stress response and spermatogenesis. Defects in these variants or differences in their relative expression levels can result in disease (7). A common mechanism of higher-order DNA organization in eukaryotes is DNA looping by proteins like cohesin and the CCCTC-binding factor (CTCF) (8, 9). Looking back in evolutionary history, eukaryotes are a sister group of the Archaea. Many archaeal enzymes and other proteins are quite similar to their eukaryotic counterparts, but often they contain fewer subunits, or they are a “simplified” version as in the case of RNA polymerase (10). Archaeal histones, for example, lack the tails for PTMs present in eukaryotic histones. Therefore, studying archaeal chromatin and transcription is partly looking back in evolutionary history from a eukaryotic perspective. On the other side of the tree of life we find the domain of Bacteria where nucleoid-associated proteins (NAPs) are the main chromatin organizers. Despite the major differences between and within the branches of the tree of life, architectural chromatin proteins use common mechanisms to organize DNA. The structural and functional properties of the main proteins are introduced in Chapter 1.

NAPs in bacteria

A major global challenge is the rise in antibiotic resistance of pathogenic bacteria such as *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus* (MRSA) (11). In this “race of arms” between humans and pathogenic bacteria, it is important to understand the evolutionary mechanisms underlying acquisition of resistance. Horizontally-transferred genes (HGTs) often contain traits that are advantageous in (for the bacteria) challenging situations (12). NAPs, such as the histone-like nucleoid structuring protein (H-NS), contribute to bacterial evolution by binding to and regulating these HGTs. Such binding can be used as a defence mechanism against bacteriophages, but can also help bacteria to build a new, advantageous, gene into a pre-existing transcriptional network. In Chapter 2, we review the H-NS-like family of proteins and discuss their shared structural and functional characteristics. We also highlight the open questions in this field such as the influence of post-translational modifications (PTMs). Many PTMs of H-NS and other important NAPs in *E. coli* have been identified in proteomics studies (13), but their functional implications are thus far unknown. Another possible layer of regulation of H-NS-like proteins is the expression of paralogs, of which we are only beginning to understand the effect they have on the DNA-binding properties of H-NS-like proteins.

An unusual member of the H-NS-like protein family is the Rok protein from *Bacillus subtilis* which we studied in Chapter 3. We found no indications for nucleoprotein filament formation besides DNA bridging and Rok is unable to complement for the loss of H-NS in *E. coli,* which is different from other H-NS-like proteins such as MvaT and Lsr2. Also its smaller paralog sRok cannot replace H-NS, but can modulate Rok's function. Interestingly, the combined expression of Rok and sRok resulted in a different transcriptional profile than for expression of the individual proteins. *Bacillus spp.* can produce a plethora of antimicrobial compounds relevant to the food industry and as therapeutic agents. As Rok is one of the regulators of these genes (14), understanding the role of these two proteins in transcriptional regulation is an important topic. To be able to benefit from the fundamental knowledge of (s)Rok in a biotechnology setting, we first need to understand how it is regulated. So far, environmental cues in the physiological range have little effect. On the other hand, the fact that Rok shows the same behavior (DNA bridging) over a wide range of environmental conditions, could make it a reliable tool for future applications.

The difference of Rok and sRok compared to other H-NS-like proteins, raises the question whether the initial inclusion of (s)Rok into the H-NS protein family was correct. Also, with the discovery of new potential H-NS-like proteins, such as MucR, GapR and EnrR (15–17), reaching a well-defined description of when a protein is an H-NS-like protein becomes very important. Until now, we have mainly used fold topology, functional properties and the types of protein-DNA complexes formed as common denominators of the protein family (Chapter 2). A protein family is a set of proteins that is evolutionarily related, reflected by related functions or their similarities in sequence or structure. H-NS-like proteins are not related by the strict definition of shared origin but rather via convergent evolution: finding a similar solution to the same evolutionary challenge using proteins unrelated by sequence and evolutionary history. One of these challenges could be horizontally transferred genes and their unregulated transcription. The solution offered by H-NS-like proteins would be xenogeneic silencing. However, this definition ignores other gene regulatory functions and the genome-organizing properties of H-NS. From a more structural perspective, the presence of an N-terminal oligomerization domain and a C-terminal DNA-binding domain are shared features of the current members of the H-NS-like protein family. Also, they all form DNA-protein-DNA bridges. With the rise of structure prediction programmes like AlphaFold, categorizing proteins based on (predicted) structure becomes easier, although experiments should be performed to verify the predictions. Previously hidden underlying patterns might in this way become visible.

One clear example is the histone protein family. The presence of histone proteins in bacteria has long been a topic of debate. In the 1970's and 1980's, proteins like HU were considered bacterial histones based on similar amino acid content and the ability to form 'nucleosome-like' structures plasmid DNA (18). Later, bacterial NAPs were instead considered functional homologues of histone proteins (such as H-NS being 'Histone-like Nucleoid Structuring protein') and until recently, histones themselves were considered absent in bacteria. However, using fold homology, people have started to note histone sequences in bacterial genomes (19). They lack the tails of eukaryotic histones, just like archaeal histones. Despite some structural difference in their histone fold compared to eukaryotic and archaeal histones, they assemble as dimers. Crystal structures show, however, that these bacterial histones do not wrap the DNA but coat the DNA as a nucleofilament. This is an unexpected deviation from the DNA binding properties of eukaryotic and archaeal histones, but it remains to be investigated if these structures are formed *in vivo* and whether they have any regulatory function. Irrespective of the answers to these questions, it is an important message that histones are more widespread in bacteria than commonly believed, and their (re)discovery might lead to new interesting insights into bacterial chromatin organization.

Hypernucleosome formation – and beyond

In the other prokaryotic branch of the tree of life, the Archaea, histone proteins are widespread (20). Most archaea encode for histone proteins that are able or predicted to form a hypernucleosome, defined as a histone multimer that wraps the DNA, creating a quasi-continuous superhelix (20, 21). Although eukaryotic histones mainly form the characteristic octameric nucleosome core, recently a more compact extended structure was found at the telomers of chromosomes, which strikingly resembles a hypernucleosome (22).

In Chapter 4 we discuss a SELEX-optimized DNA sequence, Clone20, that is bound by histone tetramers with a higher (effective) affinity than observed for hypernucleosome formation. We hypothesized that this sequence could function as a nucleation site for hypernucleosome assembly, but this is not the case. Sequences identical to the artificially optimized DNA sequences have not been identified in archaeal genomes, although one would expect that similar sequences do exist. More general characteristics for high-affinity DNA sequences have been identified, such as a TA or AA dinucleotide every ~ 10 bp, following the average helical repeat around a nucleosome (23, 24). It is unclear what the characteristics of specific positioning DNA sequences are in archaeal genomes and what is their potential function in transcriptional regulation. So far, only an indirect relationship between archaeal histones and repressive effects on transcription have been found in *Thermococcus kodakarensis* and *E. coli* (25, 26). Combining RNA-seq with a technique like ChIP-seq that can detect histone binding at a specific location, could give us a hint of which genes might be directly regulated by histones. Such model genes could help us studying the gene regulatory function of histones.

Another important aspect of hypernucleosome formation of which our understanding is very limited, is the incorporation of histone variants. Based on their (predicted) properties, speculative models have been proposed. *In silico*, different combinations of histone variants can alter the DNA binding affinity and tetramer stability (27). Thus far, most experimental studies on archaeal histones have been performed on homodimers, where slight differences between histone paralogs have been observed. For example, the hypernucleosome formed by HMfA has weaker stacking interactions and is slightly less compact than an HMfB hypernucleosome (28). Due to their high sequence similarity (84%), heterodimers are likely formed *in vivo.* Sometimes, heteromerization seems even necessary for DNA binding. For example, one of the histones of *Methanothermobacter thermoautotrophicus* OSU, HMtB, has lost its DNA binding ability in laboratory culture and can only bind DNA when forming heteromeric complexes with HMtA2 (29). Similar behaviour was observed for the two histones of *Nanoarchaeum equitans* (30). Archaea of the class Halobacteria encode double histones, where two histone folds are connected by a short linker peptide (20, 31, 32). This results in intramolecular heterodimerization. So far, no examples of such obligate heterodimerization have been observed for archaeal histones that are not physically attached as found for their eukaryotic counterparts.

In Chapter 5, we show that histone MJ1647, a variant that contains a C-terminal extension, can both bridge DNA and bind the DNA as a wrapping tetramer. Comparable to eukaryotic cohesin and other SMC proteins, the DNA bridging function of MJ1647 could potentially result in DNA looping. MJ1467 might, therewith, have a role in higher-order genome organization. Despite having a histone fold, MJ1647 does not form heteromeric complexes with the canonical histone A3 (33). This suggests that MJ1647 does not have a direct effect on the hypernucleosome formed by A3, but may act as a roadblock for hypernucleosome extension and might compete for similar DNA sequences. In this respect, MJ1647 might be more comparable to archaeal NAPs like Alba and MC1. It has been shown *in vitro* that Alba can indeed compete with histones on DNA (34), but details of the interplay between NAPs and the hypernucleosome, and the effects on gene regulation and genome organisation are still open questions.

Limitations in studying the role of archaeal histones *in vivo* mainly come from the lack of laboratory strains and accompanying genetic tools which might need to be optimized for the respective archaeal species used. Most cultured strains belong to the Euryarchaeota and TACK-superphylum (mainly Crenarchaeota), which gives us a limited view of the archaeal domain of life. Two recent articles demonstrate the culturing of two Lokiarchaeota, but also show that they are slow-growing, syntrophic organisms (35, 36). In Chapter 6, we attempted to study the DNA binding properties of histone A and B (HA and HB) from *Ca. Heimdallarchaeota* LC_3. However, obtaining the proteins turned out to be non-trivial. The successful synthesis of HB opens up opportunities of studying histones that are difficult to obtain otherwise. This technique also makes it possible to selectively incorporate PTMs at specific sites. The finding of acetylated histones in *T. kodakarensis* and *Thermococcus gammatolerans* (37) and the presence of an N-terminal tail containing several lysine resides in HA, highlights the importance of studying the effects of these modifications. Advances in culturing archaea from different phyla could provide us with more ways to study (modified) histones. Also, it could provide us with more information about abundance of different histone variants across the growth cycle and allow for studying the 3D organization of their chromosomes by chromosome conformation capture techniques like 3C and Hi-C.

Although no pathogenic archaea have been discovered yet, they might play a role in some human diseases such as periodontal disease and inflammatory bowel disease (38, 39). Most archaea, however, are extremophiles and live in the most remote places on earth, including hydrothermal vents and saline lakes. Their habitat makes it necessary for their proteins to be resistant to these extreme conditions. Also the hypernucleosome formed by archaeal histones can be regarded as an ‘extreme’ version of the eukaryotic nucleosome. Considering the theoretically endless nature of this complex, evident follow-up studies would focus on the factors that have an influence on modulating the size and stability of the hypernucleosome. Also, the interplay between hypernucleosome and the transcription machinery, and a possible specific role in transcription regulation needs to be further investigated.

Studying archaeal transcription regulation is important in the bigger scheme of nutrient cycles. As archaea play a role in methane metabolism, but also nitrogen and sulphur cycles (40–42), they might be a key player in addressing today's challenges such as global warming. On the other side, they could also provide solutions to sustainability issues. Many industrial processes involve the use of chemical compounds that are (potentially) harmful to the environment. Due to their high resistance, archaeal enzymes are good candidates to catalyse chemical reactions and thereby make industrial processes more sustainable (43).

Increasing our knowledge about genome organization of prokaryotes and the proteins responsible for this, might show us functional and structural conservation patterns previously unknown. In turn, this might explain how NAPs and histones evolved. The recent discoveries of histones in bacteria and hypernucleosome-like structures in eukaryotic telomers are good examples of evolutionary patterns that still need to be fully elucidated. Insights into the diversity of DNA organization and transcription regulation across the tree of life could help to understand the role that microorganisms play in major challenges the world faces today.

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