Solid phase synthesis strategies for generating novel chromatin model systems

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Solid phase synthesis strategies for generating novel chromatin model systems

by

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ABSTRACT

Chromatin, the native form of eukaryotic DNA, organizes the genome and regulates its use. Assembled chromatin model systems provide a powerful tool for understanding the complex structural and functional properties of chromatin. Chromatin is structurally diverse within the cell, yet current in vitro model systems do not reflect this diversity. Additionally, generating new model systems has traditionally been challenging. Here we apply the ideas of solid-phase synthesis to show that sequential ligation of DNA fragments readily generates a wide range of chromatin model systems. Using DNA fragments containing one or more nucleosome positioning sites, we generated DNA templates for assembling nucleosomal arrays containing from one to forty-eight nucleosomes, where the spacing between nucleosomes was also varied. We successfully demonstrate that the nucleosomal arrays generated are well saturated and compositionally well defined. Altogether, these strategies provide a means to vary the length and composition of chromatin models systems at the level of individual nucleosomes, and provide new tool for probing chromatin structure and function.
CHAPTER 1

INTRODUCTION TO CHROMATIN

The fundamentals of chromatin structure and function

Chromatin is the result of equal masses of proteins and genomic DNA packaged and condensed within a eukaryotic nucleus. Chromatin plays a pivotal role in regulating many cellular processes that require access to genomic DNA, such as transcription, replication and repair. Because of its importance in biology, understanding chromatin and its role in epigenetics is a topic of increasing focus in research.

The base unit of chromatin is a nucleosome consisting of approximately 147 base pairs (bp) of DNA wrapped around a histone octamer containing two copies of each histone protein, H2A, H2B, H3 and H4. Nucleosome structure is well conserved amongst eukaryotes, and has been shown to occur every 200 ± 40bp within all genomes. These nucleosomes are connected to each other via “linker DNA,” or short DNA segments. This array of nucleosomes and linker DNA are frequently referred to as the beads-on-a-string model or the primary structure of chromatin (Figure 2). It is worth noting that although chromatin’s primary structure is well characterized...
and understood, increasing numbers of histone variants are continuously being discovered which may have an effect primary structure\textsuperscript{27}.

Chromatin’s secondary structure is formed when short-range interactions between nucleosomal arrays form small 30nm fibres (Figure 2). These interactions are grossly affected by post translational modifications on histone amino acid side chains as well as the histone variants present within the core\textsuperscript{26}. The spacing of nucleosomes within the genome, more specifically DNA linker length, also has a profound effect on the secondary structure of chromatin\textsuperscript{26,28}. Because of these multiple factors, the secondary structure of chromatin has been difficult to characterize. In fact, two conflicting models of the 30nm fibre exist (Figure 3): the zig-zag model and the solenoid model. Additionally, the existence of the 30nm fibre itself has recently been questioned due to lack of reproducible evidence for it \textit{in vivo}\textsuperscript{29}. In contrast, longer range interactions between many

\textbf{Figure 2: Primary, secondary, and tertiary structure of chromatin} (Luger et al., 2012). Top: The nucleosomal array consisting of nucleosomes connected via linker DNA, also called the beads-on-a-string model. Lower-left: Secondary structure of chromatin consists of nucleosomal array interactions to form a 30nm fibre. Lower-right: Tertiary chromatin structure consists of highly ordered repeats of secondary structure and often contains architectural proteins to stabilize the structure.
nucleosomes condense to form what is known as tertiary structure, which are easily visualized fibres seen during metaphase in the cell.

Much of what we know about chromatin comes from discoveries at the nucleosomal level or from genome-wide mapping of steady state chromatin. The gap in our understanding of chromatin exists at the level of the 30nm fibre. This is known to be an instrumental regulator of DNA metabolic pathways, yet its exact structure and dynamics remains largely a mystery. There remains a need for a set of diverse in vitro chromatin model systems that can be employed to illuminate the questions surrounding the secondary structure of chromatin.
Current in vitro chromatin model systems

Currently, few in vitro chromatin model systems exist for studying higher order chromatin structure and dynamics. Widom and colleagues determined specific DNA sequences with high affinities for the histone octamer. Specifically, a positioning sequence they titled the “601” sequence showed the greatest affinity for the histone octamer. The 601 sequence has since been widely used in the field and has been incorporated into DNA templates for use in in vitro chromatin model systems. One of the most widely used template is the 601-177-$n$ template, where tandem 601 positioning sites are repeated ($n$) times with 30 bp of linker DNA between each histone protein binding site. This 601-177-$n$ DNA template has been widely used throughout the field to generate nucleosomal arrays, which is achieved by depositing purified histone protein onto the 601-177-$n$ template.

Recall that DNA linker length has been known to play a role in the secondary structure of chromatin, and the most widely used chromatin model system, the 601-177-$n$ array, has a DNA linker length of 30 bp between neighboring nucleosomes. But does this reflect how nucleosomes are spaced in vivo? Most studies have relied on MNase mapping, also called MNase-seq, to determine nucleosome positions within the genome. However, MNase-Seq is not precise and can be off by as much as 40 bp. In 2013, a method was developed to map nucleosome positions within the S. cerevisiae genome with one bp resolution. Interestingly, the results indicated that the most fre-
quent spacing between neighboring nucleosomes followed a trend of $10n+5$ bp ($n$ is number of bp), while DNA linker lengths of $10n$ were far less common (Figure 4).

Chromatin has been traditionally challenging to study, particularly in vitro, for two reasons: (1) the diverse biochemical environment chromatin is exposed to in vivo is difficult to recapitulate in a test tube and (2) chromatin has diverse and dynamic nature within the context of the cell which is difficult to capture in a model system. Our current model systems do not reflect chromatin’s structural and biochemical diversity that is present within the cell. Additionally, they have uniform linker lengths, and most often reflect the less frequent in vivo spacing of $10n$ base pairs. Additionally, most models have been limited to 12 nucleosome positioning sites. Longer nucleosomal arrays could help illuminate the details of higher order chromatin structure.

**Figure 4: Nucleosome spacing in vivo** (Brogaard et al., 2013). Single base pair resolution mapping of nucleosome positioning within the *S. cerevisiae* shows that the most frequent DNA linker lengths follow a $10n+5$ bp rule, while DNA linker lengths of $10n$ bp multiples are less common.
This study focuses on developing methods to create a more diverse set of chromatin model systems. Specifically, we chose to vary DNA linker length and number of nucleosome positioning sites within our models. Our study provides an adaptable and straightforward method for creating new chromatin model systems which are desperately needed to understand the structure and dynamics of chromatin.
CHAPTER 2

SOLID PHASE SYNTHESIS STRATEGIES FOR GENERATING NOVEL CHROMATIN MODEL SYSTEMS

Margaret K Gannon¹, Melissa J Blacketer², Michael Shogren-Knaak³

Abstract

Chromatin, the native form of eukaryotic DNA, organizes the genome and regulates its use. Assembled chromatin model systems provide a powerful tool for understanding the complex structural and functional properties of chromatin. Chromatin is structurally diverse within the cell, yet current in vitro model systems do not reflect this diversity. Additionally, generating new model systems has traditionally been challenging. Here we apply the ideas of solid-phase synthesis to show that sequential ligation of DNA fragments readily generates a wide range of chromatin model systems. Using DNA fragments containing one or more nucleosome positioning sites, we generated DNA templates for assembling nucleosomal arrays containing from one to forty-eight nucleosomes, where the spacing between nucleosomes was also varied. We successfully demonstrate that the nucleosomal arrays generated are well saturated and compositionally well defined. Altogether, these strategies provide a means to vary the length and composition of chromatin models systems at the level of individual nucleosomes, and provide new tool for probing chromatin structure and function.
Introduction

In eukaryotic organisms, genomic DNA is sequestered as chromatin, a complex structure containing double stranded DNA and nuclear proteins. The simplest structural unit of chromatin is the nucleosome, in which DNA is wrapped 1.65 times around a histone octamer, a protein spool comprised of two copies each of histones H2A, H2B, H3, and H4 (1). The majority of genomic DNA is wrapped into nucleosomes, and their arrangement varies throughout the genome, with some regions demonstrating highly regular spacing of nucleosome and with most others showing irregular nucleosomal spacing (2,3).

While the structural and functional properties of single nucleosomes are relatively well defined (4-7), the effect of arranging many nucleosomes in tandem is not nearly as well understood. Arrays of nucleosomes can undergo several different structural transitions, including intra-array folding that decreases the length of chromatin fibers (8,9), and cross-strand interactions that reversibly associate chromatin fibers (9,10). However, the precise nature of these structures, the strength and dynamics of interactions, and the crucial factors underlying them remains controversial (11,12). Additionally, arrays of nucleosomes appear to have functional effects beyond individual nucleosomes, including restricting access to DNA between nucleosomes (13), stabilizing nucleosomes to octamer loss (14), and changing the ability of DNA to loop (15). However,
other function effects are likely, and the basis and generality of the observed effects to a broader range of nucleosomal arrangements and nuclear factors remains to be explored.

To address the structural and functional effects of systems containing many nucleosomes, a key strategy has been to generate chromatin model systems consisting of a tandem array of uniformly spaced nucleosomes. Generally, this has been accomplished by depositing histone octamers onto a DNA template containing multiple head to tail repeats of a strong nucleosome positioning sequence (9,16). These DNA templates are often generated by cloning multiple copies of a single nucleosome positioning sequence into a receiving vector (9,17), and while this strategy has been successfully utilized to generate DNA templates with different lengths and nucleosomal spacing, it is often time consuming, and difficult to precisely control the length and composition of the template.

To address the limitations in generating new DNA templates for chromatin model-system assembly, we were interested in exploring a solid-phase synthesis strategy. Such an approach has proven to be highly effective method for synthesizing biological polymer, such as DNA and peptides. In solid phase synthesis, a biopolymer chain is built off a bead and synthesized directionally. By incorporating monomers onto the chain one a time, the length and precise sequence of the polymer at every position can be controlled. Furthermore, because excess of monomer can be used at every step, and because uncondensed monomers can be easily removed by washing the bead between
steps, each addition step can potentially proceed with high yield with little misincorporation, further controlling the composition of the resulting product. Here, using different DNA monomers, we demonstrate our ability to generate a range of novel chromatin model systems via a solid-phase ligation strategy.

Materials and Methods

Preparation of ligatable DNA fragments

To make the non-palindromic (NPD) DNA monomers, NPD-172-1 and NPD-172'1, a 601-177-1 DNA fragment (19) was used as a template for PCR amplification with the following primers: 172BglIF: 5’-GCATGCTGCA GCCAGAATGGGTACATG-CACAGGATGTATATC-3’ and 172Bgl1IR: 5’-GCATGCTGCAGCCACCTTGGCGGCCGCCCTGGAGAATCCCG-3’ to generate NPD-172-1, and 172'BglIF: 5’- GCATGCTGCA GCCAGAATGGGTACATG-CACAGGATGTATATC-3’ and 172'Bgl1IR: 5’-GCATGCTGCAGCCACCTTGGCGGCCGCCCTGGAGAATCCCG-3’ to generate NPD-172'-1. Amplified products were digested with PstI and cloned into the pRS315 vector at the PstI site creating plasmids pRS315-172-1 and pRS315-172'-1. Fragments NPD-172-1 and NPD-172'-1 were excised by BglII digestion of their respective plasmids and gel purified.
Biotinylated, double strand DNA, “adapter” fragments with non-palindromic sticky ends were made by annealing (20) the following oligonucleotides (IDT), where Bio refers to biotin and 5PCBio refers to photocleavable biotin: ADT-BglI’: 5’-5PCBio-GGCCGCCTGCAGATATCGAA-3’ and 5’-Phos-GATATCTGCAGGCAGGCGCC-3’. ADT-BstXI’: 5’-5PCBio-ACGACGGCCAGTGAACCACGATT-3’ and 5’-Phos-GTG-GTTCACTTGCCGTCGT-3’

Double stranded, DNA, “cap” fragments with non-palindromic sticky ends were made by annealing (20) the following oligonucleotides (IDT): CAP-BglI: 5’-GATATC-GATCCAATTAT-3’ and 5’-ATAATTGGATCCGATATCttc-3’. CAP-BglI’: 5’-GATATCG-GATCCAATTAT-3’ and 5’-ATAATTGGATCCGATATCCCT-3’.

Assembly of Nucleosomal Substrates for Ligation

Recominant Xenopus laevis histones were expressed, purified, and then assembled into histone octamers according to standard protocols (21). Histone octamers were deposited onto 12mer DNA template, NPD-177-12, via step-wise salt deposition using previously described methods (19,20).

DNA Solid-Phase Ligation

The 601-172-12 nonpalindromic DNA template, NPD-172-12, was created as follows: 1.14 pmoles of biotinylated nucleotide adapter, ADT-BglI’, was immobilized onto
either 12µl or 120µl of magnetic streptavidin beads (NEB) (20). 2.25x-fold of fragment NPD-172-1 was ligated to the adapter for 2 hours at RT in the presence of 2x of Mighty Mix Ligation Mix (Takara) in a total reaction volume of 10µl. The beads were then washed 2x with 100µl of 1X Ligation Buffer to remove any excess, unligated fragment. Ligation and washes were continued, alternately attaching fragment NPD-172’-1 and NPD-172-1. After twelve rounds of ligation, 2.25x CAP-BglII was ligated. The ligated and “capped” fragments were photocleaved from the beads in 20µl of 1X NEB 3.1 digestion buffer (NEB) for 10 min with exposure to 312 nm UV light. 20 units of PstI and BamHI were added to digest for 2 hours. The released fragments containing various numbers of 172 bp repeats were gel purified and cloned into p601X (14) to create plasmids p601X-172-8, p601X-172-10, and p601X-172-12. DNA templates NPD-172-8, NPD-172-10, and NPD-172-12, respectively, could be excised with PstI/BamHI digestion and gel purified.

Gel Analysis of Nucleosomal Arrays

Nucleosomal arrays were digested with restriction enzymes to determine correct assembly, saturation and stability. The general protocol for digestion was as follows: 60ng of 601-172-12 and 601-177-12 nucleosomal arrays were combined with 20 units of BglII or Scal, respectively, in a total digestion volume of 20µl. The reaction incubated at 37°C for 2 hours. The nucleosomes and free DNA generated by restriction digestion was
characterize by 4% native PAGE analysis in 0.5X TBE, according to standard protocols (16).

Multi-angle light scattering

The molecular weight of nucleosomal arrays was determined by couple size exclusion chromatography and multi-angle light scattering as previously described, with the following changes: In the protein conjugate analysis used to determine array saturation, the array was decomposed into free DNA monomers and mononucleosome components, using the following parameters determined from free DNA monomer and mononucleosome model systems: DNA $\varepsilon_{260}$ of 20 ml•mg$^{-1}$•cm$^{-1}$ and $dn/dc$ of 0.1269 ml•g$^{-1}$. Mononucleosome $\varepsilon_{260}$ of 17.74 ml•mg$^{-1}$•cm$^{-1}$ and $dn/dc$ of 0.1269 ml•g$^{-1}$.

Results

Solid phase ligation strategy

The overall scheme for the solid phase ligation is shown in Figure 1. In this strategy, a biotinylated adapter DNA with a non-palindromic sticky end is attached to a solid support coated with streptavidin. A monomer containing DNA with a complementary non-palindromic sticky end is then added with a DNA ligase to condense the first monomer to the bead. These monomers can be DNA fragments containing a single nu-
cleosome positioning site or DNA fragment containing multiple nucleosome positioning sites. Ligation steps are repeated until the desired product is generated. The product is then photocleaved or enzymatically cleaved from the resin via a unique restriction enzyme site contained in the adapter DNA. The product is a DNA template (Figure 1A) that can be cloned, characterized, and used for assembly of nucleosomal arrays.

For the adapter, we chose the biotin-streptavidin interaction because of its strength, and we found that when we added adapter at amounts sub-stoichiometric relative to streptavidin, we got complete binding (data not shown). We did not try to maximize the adapter den-
sity on the beads, because as discussed subsequently, we found that yield of full-length product was greater with a lower ratios of adapter to bead. A photocleavable linkage and a restriction enzyme site were included in the adapter to ultimately liberate ligated products from the bead.

The adapter DNA contains one non-palindromic sticky end to facilitate annealing and ligation. The monomers contain two non-palindromic sticky ends. One end is complementary to the terminal end of the growing, bead-bound product, and the other contains a non-palindromic sticky end that is incompatible with ligation to the other sticky end pairs, but can be ligated to a subsequent fragment. This arrangement of non-palindromic sticky ends means that only two types of fragments (labeled Fragment A and B in Figure 1) are required to perform multiple cycles of polymer condensation, reducing the number of monomers that need to be made. Non-palindromic sticky ends are necessary because monomers with palindromic ends can self-ligate, both reducing the efficiency of ligation to the bead-bound product, and creating the potential for incorporation of more than one monomer into the growing chain per ligation step. In test reactions in solution we found that not only were these non-palindromic sticky ends efficient substrates for ligation, but also that self-ligation of the monomers was not observed (data not shown).

A feature of solid phase synthesis strategies is that reagents can be readily washed away from the bead-bound polymer chain between ligation steps. This is ad-
Figure 2. Synthesis of a DNA template with reduced spacing between nucleosome positioning sites and characterization of a nucleosomal array assembled from it. (A) Depiction of the two 172 bp, single nucleosome-positioning DNA monomers used for ligation, NPD-172-1 and NPD-172’-1. Both monomers contain ends generated by BglII digestion, but four different overhangs are generated. The relative compatibility or incompatibility of these non-palindromic overhangs toward annealing and ligation is depicted schematically by the shape of the ends. (B) Gel electrophoresis analysis of the product of 12 rounds of 601-172-1 DNA monomer ligation, either optimized for full-length product (lane 1), or for diversity in product lengths (lane 2). (C) Native gel analysis of the crude products of array assembly for one 601-172-12 assembly and three 601-177-12 assemblies. ‘R’ indicates the molar ratio of histone octamer to DNA 601 positioning sites in the 12-mer template. 0.3 molar equivalents of a weaker carrier DNA positioning fragment are present during assembly. (D) Native gel analysis of the digestion products of purified 601-172-12 and 601-177-12 arrays using BglII or Scal, respectively. (E) Size exclusion chromatography and multi-angle light scattering analysis of purified arrays. The UV-Vis elution profiles of the 601-172-12 and 601-177-12 arrays with R=0.9 are depicted with solid red and blue lines, respectively, with associated absolute molecular weights indicated as dotted lines. Representative data from one of three trials for each array is shown.
vantageous for several reasons. First, by washing away unincorporated monomer after a ligation step, that monomer will not be present in the subsequent condensation reaction to cross-react. Additionally, the ease of removing unincorporated monomer allows an excess of monomer to be used during ligation, which can drive help drive the reaction to completion. In our DNA ligation reactions we typically used 2.25-fold amount of monomer relative to the growing chain. These amounts gave a good tradeoff between having an excess of reactant and consuming reagents. Stringent wash conditions between ligation steps are also enforced to prevent undesired cross-reactivity.

**Synthesis of nucleosome assembly templates with different intranucleosome spacing**

Our first test of the solid-phase ligation strategy was generating new nucleosomal array DNA templates with shorter DNA distances between nucleosome positioning sites. A commonly utilized DNA template for generating nucleosomal arrays is the 601-177-12 template (9). This template consists of 12 copies of the strong, single positioning sequence, ‘601,’ originally developed for assembling well-behaved mononucleosomes (22). The ‘601’ positioning site wraps 147 bp of DNA around a histone octamer, and thus has 30 bp between nucleosomes. We were interested in generating a new nucleosomal array template, 601-172-12, in which the spacing between nucleosomes was reduced by five bp, approximately half a turn of a B-form DNA helix. To do so, we needed two different 601-172-1 monomers that could be used in the solid-phase ligation (Figure 2A).
These two fragments with different non-palindromic sticky ends were generated by PCR amplification from a 601-177-1 template, cloned into a plasmid in single copy, and then isolated after restriction enzyme digestion.

Using these monomers, we performed twelve rounds of ligation, building off of an adapter DNA bound to the solid support through a biotin-streptavidin interaction (Figure 1A). This product was photocleaved from the beads and analyzed by gel electrophoresis (Figure 2B, lane 1). While intermediates were present in the cleaved product, the predominant species observed migrated as expected for the 601-172-12 product. Interestingly, in optimizing the conditions for ligation, we found that, while a higher density of DNA adapter to bead still generated the desired 12-mer product, a larger amount of ligation intermediates were also present (Figure 2A, lane 2). Because our goal was to ultimately clone the desired template, we viewed the presence of ligation intermediates as a way of readily generating array templates of different lengths. As proof of this principle, we isolated bands corresponding to 8, 10, and 12 ligations and cloned them into a vector. DNA sequencing confirmed that we had generated 601-172-8, 601-172-10, and 601-172-12 templates.

**Assembly and analysis of nucleosomal arrays on synthesized templates**

To generate nucleosomal arrays from the 601-172-12 template and compare them to 601-177-12 arrays, we employed standard techniques of salt-step dialysis (9,16). In
this approach, recombinant histone octamers are mixed with template at high salt concentrations (2M NaCl), and then the salt is dialyzed away in several steps to ultimately deposit octamers onto the nucleosome positioning sites. To aid in proper deposition, a weaker mononucleosome position sequence, referred to as carrier DNA, is also added. A key issue in comparing assembled nucleosomal arrays is ensuring that the same numbers of octamers have been deposited, as array properties will vary as a function of the nucleosomal saturation of the template. In theory, similar degrees of array saturation should occur if the same molar stoichiometry of octamer, nucleosomal sites, and carrier DNA are present during assembly. However, additional means of confirming this saturation are necessary. We confirmed similar array saturations a number of different ways. During assembly, we used a molar ratio of histone octamer to nucleosomal sites (‘R’) of 0.9 for the 601-172-12 template, and ‘R’ values of 0.8, 0.9, and 1.0 for assembly onto the 601-177-12 template. In these experiments, the carrier DNA is added at a molar ratio of 0.3, and is expected to become occupied after filling the 601 sites in the 12mer template. Indeed, if we analyze the crude assembly products of the various arrays on a 4% native PAGE gel, where we can resolve free carrier DNA from octamer occupied carrier, we observe that for the 601-177-12 assemblies, no carrier mononucleosome is observed until a small amount is visible at an ‘R’ value of 1.0 (Figure 2C). 601-172-12 arrays assembled with R= 0.9 do not show any carrier mononucleosome and are consistent with the 601-177-12 array with the same ‘R’ value.
To more directly compare the saturation of the arrays, we exploited the fact that there are BglII or Scal restriction sites between every nucleosome positioning sequence in the 601-172-12 (Figure 2A) and 601-177-12 arrays (9), respectively. Gel analysis of the digestion products of the purified 601-177-12 arrays shows that with increasing ratios of octamer to nucleosome positioning sites, the amount of DNA that is not wrapped as a nucleosome decreases, and very little of this DNA is present when arrays are assembled with an equal ratio of octamer and nucleosome positioning sites (Figure 2D). For the 601-172-12 array with R=0.9, the digestion pattern looks similar to the analogous 601-177-12 array, suggesting that both arrays have a similar level of nucleosomal saturation.

As another way to assess the saturation of the arrays, we performed size exclusion chromatography with determination of the absolute molecular weight of the arrays by multi-angle light scattering, SEC-MALS (Figure 2E). These analyses further indicate that the 601-172-12 and 601-177-12 arrays with R=0.9 are well matched in terms of saturation, having molecular weights of 2.48 +/- 0.3 MDa and 2.49 +/- 0.6 MDa, respectively. These molecular weights correspond to arrays saturations of 11.1 and 10.9 nucleosomes/array, respectively, and correlate well with the saturation expected for arrays prepared with an R of 0.9.
Synthesis of longer nucleosome assembly templates

In addition to shorter nucleosomal array templates, the solid-phase ligation strategy can be used to generate DNA templates for making longer nucleosomal arrays. For example, DNA templates containing twelve nucleosome-positioning sites could be used as the ligation monomer to generate new templates containing multiples of twelve nucleosome-positioning sites (Figure 1A). As proof of this concept, we started with standard 601-177-12 DNA templates, and through cloning and PCR manipulation, generated two new 601-177-12 templates containing...
two different pairs of non-palindromic sticky ends (Figure 3A). With these monomers, we performed multiple rounds of ligation. Shown in Figure 3B are the products of a ligation experiment where we optimized conditions to generate dimeric and tetrameric ligation products. In this experiment, the final ligation step was to add a DNA cap (Figure 1A) with an overhang (BglII, Figure 3A) compatible with the non-palindromic end of the 601-177-24 and 601-177-48 products. These 24mer and 48mer products were then digested with restriction enzymes that recognize sites in the DNA cap and DNA adapter (Figure 1A), and cloned into a plasmid. A 36mer templates was generated in a similar manner (data not shown).

**Assembly and analysis of nucleosomal arrays on longer synthesized templates**

We assembled nucleosomal arrays onto 601-177-24, 601-177-36, and 601-177-48 DNA templates by salt step dialysis as described above for the 601-172-12 arrays. To characterize the arrays for proper assemble and desired saturation, we again employed restriction digestion analysis and SEC-MALS analysis. Similar to the 601-172-12 arrays, ScaI digestion was used to cut the assembled arrays into fragments to assess to what extent nucleosome binding sites were assembled into nucleosomes or still existed as free DNA. However, because of the way that the 12mer ligation monomers were generated, not every nucleosome positioning site was flanked by a ScaI sites (Figure 3A). Thus, while the majority of digested fragments were expected to be mononucleosomes, some
dinucleosomes and a small amount of tetranucleosomes were also expected. Native gel analysis of the digestion products (Figure 3C), revealed such a distribution of products for each of the three arrays. Importantly, as the arrays were assembled with a molar ratio of 1:1 octamer to nucleosome positioning site, we expected to observe very little free 601-177-1 DNA fragments relative to 601-177-1 mononucleosomes, and indeed, that was the case, suggesting that the arrays had the desired saturation and had assembled properly. To further support this we performed SEC-MALS analysis (Figure 3D). The arrays, especially the 48mer array, eluted near the void volume of the size exclusion column and prevented us from fully resolving the arrays from potentially larger assembly products. Nonetheless, the absolute molecular weights associated with each elution peak (5.16 +/- 0.20 MDa, 7.60 +/- .01 MDa, and 10.8 +/- 2.0 MDa, for the 24mer, 36mer, and 48mer arrays, respectively) were consistent with saturated arrays (23.5, 34.0, and 51.0 nucleosomes/array, respectively). Thus, our data indicates that our longer DNA templates function well for assembling longer nucleosomal arrays.

**Discussion**

In this study, we have explored to what extent a solid-phase ligation strategy can be used to generate reagents for studying chromatin model systems. Using DNA fragments as monomers, we have shown that we can generate nucleosomal array templates with different number of nucleosome positioning sequences as well as different spacing
between them. The ligation process is relatively efficient, but incomplete ligation products are observed. For example, with the generation of 601-172-12 array template, the 12mer product is the predominant product, but some 11mer product and lesser amounts of smaller species are seen (Figure 2B, lane 1). To achieve this extent of reaction, we had explored a number of different experimental parameters, including ligation times, ligase sources, and reagent amounts and concentrations. One important parameter we have found to be important for efficient ligation is the initial density of the DNA adapter on the beads, where lower ratios of adapter DNA to beads generally result in more efficient ligation. A striking example of this difference is seen in comparing lanes 1 and 2 of Figure 2B, where a higher adapter DNA to bead ratio was used in lane 2. We suspect this difference ultimately results from the charge density present near the bead surface. With a greater initial surface charge density, we believe that annealing and ligation of subsequent negatively charged DNA fragments is made more difficult, where subsequent rounds of ligation can potentially compound this issue. To some extent, the ion composition of the reaction could also modulate this electrostatic repulsion, as it has been shown that the efficiency of immobilization of DNA to beads is facilitated by increases in solution ionic strength (23). However, because the ligase itself is sensitive to ionic conditions, dramatic changes in ion composition may not be tolerated.

While we found that we could not drive twelve rounds of ligation to completion, we found that complete reaction was not necessary. In fact, we found that we could take
advantage of the multiple products generated by incomplete ligation to clone DNA templates with different number of nucleosome positioning sites (Figure 2B lane 2, and Figure 3B). This strategy offers a rapid way of systematically generating nucleosomal array templates that vary in their number of nucleosome positioning sites.

Cloning the ligation product into a plasmid provides a means of storing and amplifying the desired templates. However, we have found that the repetitive nature of the templates can make them prone to rearrangements, and that it is important to characterize the templates generated. This recombination may also play a factor in our observation that our transformation efficiency for the 24mer, 36mer, and 48mer templates was low. Potentially, even longer templates may be even more difficult to clone. A potential alternative for generating any of the templates is to use a DNA adapter and cap containing unique primer sites, allowing PCR to be used to generate larger amounts of template from ligation products.

Altogether, we feel that the techniques described for solid-phase DNA ligation significantly broaden the types of chromatin model systems that can be readily accessed, and that such systems will drive a better understanding of chromatin structure and function.
References


CHAPTER 3

THE VAST APPLICATIONS OF NOVEL CHROMATIN MODEL SYSTEMS

Biophysical characterization of new chromatin model systems

Our method of solid-phase synthesis for generation of new chromatin model systems will greatly diversify the available tools for studying chromatin dynamics. Using our new method, we were able to successfully engineer a 601-172-12 DNA template (25 bp DNA linker length between nucleosomes) that was ultimately used for assembling nucleosomal arrays. This model system is of particular importance due to the discovery that the 25 bp DNA linker length is three times more likely to appear within the yeast genome than a linker length of 30 bp represented by the 601-177-12 model. It would be of interest to investigate why this $10n+5$ bp pattern is preferred in the cell compared to the $10n$ pattern (Figure 4 in introduction).

To begin to answer this question, we can use biophysical techniques to investigate the differences between the two model systems. One avenue our lab has begun to investigate is using sedimentation velocity experiments to compare the 601-177-12 and 601-172-12 models. The sedimentation coefficient “S” tells us information about species mass and shape. Unfolded proteins or proteins with highly elongated shapes will experience more hydrodynamic friction, and thus will have smaller sedimentation coefficients than a folded, globular protein of the same molecular weight. Recall our
601-172-12 and 601-177-12 nucleosomal arrays have nearly equal molecular weights that have verified qualitatively though digestion and quantitatively via SEC-MALS analysis (Figure 2D and E). Preliminary sedimentation results show that the assembled 601-172-12 template has an S value that is smaller on average than the 601-177-12 array (Figure 1). These results indicate that the 601-172-12 array may have a more open conformation, where as the 601-177-12 array displays a more compact shape in solution with low ionic strength. Additional studies can be done using sedimentation velocity to assess each model’s ability to self associate as a function of mono- and divalent cations. This is a unique characteristic to nucleosomal array systems—multiple tandem nucleosomes can undergo reversible inter-strand self-association in the presence of mono- and divalent cations, where increasing array length increases ability to self-associate$^{10,14}$. Such sedimentation velocity experiments could provide a wealth of information about the differences in the two models, such as their capacity to form chromatin fibres. These results could help
elucidate why the $10n+5$ bp configuration is preferred in the cell to the $10n$ organization.

**Solid-phase synthesis of additional novel chromatin model systems**

In addition to the generation of DNA templates with different numbers of, and spacing between nucleosome positioning sites, this solid phase ligation strategy could also be used to generate other kinds of DNA templates. Typical nucleosomal array templates contain nucleosome-positioning sites that are uniformly spaced. However, this ligation strategy could be used to generate templates where the distance between nucleosomes varies between nucleosomes, a situation that is more commonly observed *in vivo* than uniform spacing. Also, templates can be synthesized in which the DNA binding site for a specific nucleosome is different from the other DNA binding sites within
the array, which can be used for applications such as site specific introduction of restriction sites or fluorescent FRET pairs\textsuperscript{13,24}.

In addition to ligating DNA fragments to make templates for chromatin model system assembly, the solid phase ligation strategy also offers the potential to directly generate nucleosomal arrays by polymerizing nucleosome monomers on the solid support (Figure 2). Solid-phase nucleosome ligation offers the ability to control octamer composition at specific positions in the nucleosomal array. In addition to placing well-defined gaps between nucleosomes, this technique provides a way to target histones with post-translational modifications or biophysical reporters, or histone variants to desired locations within a chromatin model system. Our lab has already made headway in optimizing this method.

Our techniques describing a solid-phase ligation strategy will provide a means of expanding the types of chromatin model systems for \textit{in vitro} studies. We feel that these systems will be a useful tool in the epigenetic field, and will help elucidate questions surrounding chromatin structure, function and dynamics.
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