The translational placement of nucleosome cores in vitro determines the access of the transacting factor suGF1 to DNA

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INTRODUCTION

The sea urchin G-string binding factor (suGF1) is one of several proteins that bind sequence-specifically to oligo(dG·dC) motifs, frequently present upstream of eukaryotic genes. In this study we investigate the interaction of suGF1, purified to near homogeneity, with its oligo(dG·dC) binding site in a reconstituted nucleosome core in vitro. We show that the in vitro reconstitution of a 214 bp fragment containing a suGF1 binding site results in the appearance of five distinct nucleosome core species. These species contain the histone octamer in an identical rotational setting but in different translational frames. The resulting different nucleosomal locations of the suGF1 binding site in the five core species are shown to modulate the ability of suGF1 to bind to nucleosomal DNA, even though the rotational setting of the DNA in the nucleosome cores maximally exposes the suGF1 binding site. We propose that a direct protein–protein steric clash between suGF1 and the histone octamer is the most likely determinant in modulating the binding of suGF1 to its nucleosomally wrapped binding site. This result suggests that in vivo suGF1, like TBP, NF1 and heat shock factor, may require a complementary nucleosome disrupting activity or that suGF1 binds to free nascent replicated DNA prior to nucleosome deposition.

ABSTRACT

The sea urchin G-string binding factor (suGF1) is one of several proteins that bind sequence-specifically to oligo(dG·dC) motifs, frequently present upstream of eukaryotic genes. In this study we investigate the interaction of suGF1, purified to near homogeneity, with its oligo(dG·dC) binding site in a reconstituted nucleosome core in vitro. We show that the in vitro reconstitution of a 214 bp fragment containing a suGF1 binding site results in the appearance of five distinct nucleosome core species. These species contain the histone octamer in an identical rotational setting but in different translational frames. The resulting different nucleosomal locations of the suGF1 binding site in the five core species are shown to modulate the ability of suGF1 to bind to nucleosomal DNA, even though the rotational setting of the DNA in the nucleosome cores maximally exposes the suGF1 binding site. We propose that a direct protein–protein steric clash between suGF1 and the histone octamer is the most likely determinant in modulating the binding of suGF1 to its nucleosomally wrapped binding site. This result suggests that in vivo suGF1, like TBP, NF1 and heat shock factor, may require a complementary nucleosome disrupting activity or that suGF1 binds to free nascent replicated DNA prior to nucleosome deposition.

INTRODUCTION

The packaging of DNA into nucleosomes in eukaryotic nuclei poses a severe steric constraint on proteins that functionally require direct access to the DNA molecule (reviewed in 1). This implied ability of chromatin to modulate the accessibility of DNA to DNA binding proteins may in some cases regulate protein–DNA interactions (reviewed in 2). Several studies (3,4) suggest that nucleosome disrupting activities, such as that associated with the Swi–Snf complex (5) may be required to expose DNA binding sites to proteins in a regulated fashion. It has also been proposed that some proteins may bind to their recognition sequences shortly after DNA replication, prior to assembly of the nascent DNA into chromatin (6). Some proteins, such as Gal4p (7), Sp1 (8), the glucocorticoid receptor (9) and the Myc–Max heterodimer (10), have the ability to bind to their recognition sequences within a nucleosome in vitro independent of a separate complementing nucleosome disrupting activity. In contrast, proteins such as Pho2p require the initial binding of Pho4p to expose appropriate binding sites in a progressive manner (11). Several more proteins, such as TBP (12), NF1 (9) and heat shock factor (13), have been shown to be unable to bind to their recognition sequences in nucleosomes and may require the disruption of a nucleosome(s) in a regulated and targeted fashion.

Sea urchin G-string binding factor (suGF1) is a protein of 59.5 kDa that is present in the nuclei of at least the 4–24 h developmental stages of the sea urchin Parechinus angulosus embryo and binds sequence-specifically to double-stranded oligo(dG·dC) (14). Oligo(dG·dC) motifs are frequently found upstream of several unrelated eukaryotic genes (15,16) and have been implicated in gene regulation in several studies (15,16). Proteins that bind to these oligo(dG·dC) motifs have been detected in several different organisms and tissues (16–19) and it was suggested that suGF1 may be a member of a family of G-string binding proteins (14). suGF1 was shown to bind to the chicken BGP1 oligo(dG·dC) recognition sequence upstream of the chicken β-A-globin gene, producing a DNase I footprint indistinguishable from that of BGP1 (14). Similar binding was demonstrated to a positive regulatory (dC6G6) cis-element upstream of the sea urchin aboral ectoderm-specific gene LpS1β (14), which is regulated by a protein related to the mouse IF1 factor (20) involved in the regulated expression of the mammalian α1(I) and α2(I) collagen genes. Recent evidence shows that the Lps1 promoter-proximal G-string, which binds suGF1 in vitro (14), lies within an extracellular matrix response element and suggests that suGF1 is regulated by the extracellular matrix (C.Thomlinson, personal communication).

We have previously reported the purification of suGF1 to 85% homogeneity, when it is present as a single band on a silver stained SDS–PAGE gel (14) and presented the results of footprinting and methylation interference studies of this purified protein (21). In this paper we report on the interaction of purified suGF1 with its nucleosomally wrapped recognition sequence in vitro. This study

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was undertaken as a first step in addressing the functional interaction of suGF1 with chromatin in vivo. Here we reconstitute a fragment from the histone H1–H4 intergenic spacer of the sea urchin Psammechinus miliaris early histone gene battery. This fragment contains a high affinity binding site for suGF1 (14) and was previously shown to position a nucleosome core in a well-defined rotational and translational setting in a 2 kb plasmid in vitro (22). These properties of the DNA fragment constitute an ideal model system to investigate the effect of the histone octamer on the ability of suGF1 to recognize and bind to its recognition sequence in a nucleosome core in vitro.

MATERIALS AND METHODS

Nucleosome core reconstitution

Long chromatin, stripped of histone H1/H5, was prepared from chicken erythrocytes as described by Drew and Calladine (23) and stored at −20°C at a concentration of 1 mg/ml in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA (TE (pH 8.0)) containing 50% v/v glycerol. Stored chromatin fractions were prepared for reconstitution by dialysis against TE (pH 8.0) at 4°C, concentrated to 1 mg/ml in a Centricon-10 centrifuge (Amicon) and the proper core histone stoichiometry and lack of proteolysis confirmed on a 15% SDS–polyacrylamide gel by an established method (24; data not shown). Nucleosome cores were reconstituted onto a gel-purified (25) 214 bp AspI–XbaI fragment (AX fragment), obtained from plasmid pH2 (26). The AX fragment contains a section of the spacer between the histone H1 and histone H4 genes of the sea urchin P. miliaris early histone gene battery (27). In this paper the sequence is numbered relative to the mRNA cap site (14), which places the AspI site at position −449 and the XbaI site at position −233. The DNA fragment was 3′-radiolabeled by an established procedure (25) at either the AspI–XbaI-generated end with [α-32P]dCTP or at the XbaI-generated end with [γ-32P]ATP and the Klenow fragment of T4 DNA polymerase (sp. act. 10⁸ c.p.m./µg). Alternatively, the AX fragment was labeled at a unique 5′-end to a sp. act. of 10⁷ c.p.m./µg with [γ-32P]ATP and T4 polynucleotide kinase after XbaI or AspI 214 bp cleavage and dephosphorylation, followed by XbaI or AspI 214 bp cleavage and gel purification respectively. Nick-translation of the AX fragment by the incorporation of [α-32P]dCTP (10⁶ c.p.m./µg) was performed by an established procedure (25).

The radiolabeled AX fragment (typically 1 µg) was reconstituted into a nucleosome core by high salt exchange of the histone octamer from 50 µg H1/H5-stripped chromatin, as described by Drew and Calladine (23). The reconstituted mixture was recovered in a final volume of 1 ml, concentrated to 5-fold in a Centricon-10 centrifuge and separated from the long chromatin histone source by isokinetic centrifugation at 36 000 r.p.m. (Beckman SW40 rotor) for 16 h at 4°C on a 5–20% w/v sucrose gradient in 10 mM Tris–HCl, pH 8.0, 0.25 mM EDTA, 0.1 mg/ml BSA and 50 µg/ml sheared herring sperm DNA (29). The electrophoretic mobility of the cores was unaffected by this procedure (data not shown).

The stoichiometry of the core histones in the reconstituted complexes was determined after the preparative electrophoresis of specific core samples. Regions of the gel containing the core complexes of interest were identified by autoradiography of the wet gel, cut out as a slice and soaked in 2 vol SDS–PAGE sample application buffer containing 3% w/v SDS. The samples were incubated at 60°C for 30 min, followed by electrophoresis on a 15% SDS–PAGE gel with a 4% polyacrylamide stacking gel header (24). The electrophoretically resolved histones were visualized by non-ammoniacal silver staining according to an established procedure (25).

Reconstitution of the suGF1–core ternary complex

Small amounts (typically 0.5–5 ng) of purified suGF1 (generously supplied by D.Patterton), obtained from 14 h embryos of P. angulosus, were incubated with 2 ng reconstituted 32P-labeled AX fragment in buffer A [150 mM KCl, 16 mM Tris–HCl, pH 7.5, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.01% v/v NP-40, 0.4 mg/ml BSA and 60 µg/ml poly(dI-dC)] in a final volume of 100 µl at 4°C for 30 min. The resulting complexes were further treated as indicated in the text. The sequence specificity of suGF1 binding to a nucleosome core was determined by including a double-stranded oligonucleotide with a suGF1 binding site (specific competitor 5′-GATCAGAGAGGGGGGGGGGAGGAGAAATT-3′) or without the binding site (non-specific competitor 5′-GATCTTCTGCACCTCCTCAGCGGTACCTGGACT-3′) (14) in the reaction mixture described above at concentrations indicated in the legend to Figure 2. The specific or non-specific oligonucleotide was added prior to addition of suGF1 to the core preparation. These complexes were further treated as described in the text.

Nuclease and hydroxyl radical digestions

Nucleosome cores purified on a sucrose gradient were incubated in the presence or absence of suGF1 as described above and digested at 4°C in 100 µl buffer A with 0.8 U/µl DNase I or with hydroxyl radicals as described (21). Following digestion, the nucleoprotein complexes were resolved on a native 4% polyacrylamide gel-purified reconstituted cores (100 ng) in 1 ml volumes of 50 mM NaCl, 10 mM Tris–HCl, pH 8.0, 3 mM MgCl₂, 1 mM 2-mercaptoethanol, 50 µg/ml sheared herring sperm DNA and 0.1 mg/ml BSA at 37°C. At the times indicated in the legend to

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of 10 mM Tris–HCl, pH 8.0, 1 mM CaCl₂, 0.25 mM EDTA, purified from a nucleoprotein gel was performed in 200 µl volumes of 10 mM Tris–HCl, pH 8.0, 1 mM CaCl₂, 0.25 mM EDTA, 50 µg/ml sheared herring sperm DNA and 0.1 mg/ml BSA with 0.4 U MNase/µg DNA at 20°C as described by Dong (31). The digestions were terminated as for the exo III digestions above and the DNA purified and analyzed on 12% polyacrylamide–8 M urea gels as described above.

Limit micrococcal nuclease (MNase) digestion of nucleosome cores reconstituted with 10 ng nick-translated AX fragment and purified from a nucleoprotein gel was performed in 200 µl volumes of 10 mM Tris–HCl, pH 8.0, 1 mM CaCl₂, 0.25 mM EDTA, 50 µg/ml sheared herring sperm DNA and 0.1 mg/ml BSA with 0.4 U MNase/µg DNA at 20°C as described above.

The presence of multiple nucleosome core species following reconstitution of the 214 bp AX fragment is most likely due to different stable translational settings of the octamer and were termed core I, II and III in order of increasing sedimentation rate, as indicated in Figure 1A. The presence of properly formed nucleosome cores in the core I and core II preparations were also demonstrated by the limit MNase digestion of these samples, which resulted in the appearance of the expected core-length fragments of ~150 bp (data not shown). Appropriate fractions from the sucrose gradient were pooled and these purified core preparations used in subsequent experiments described below. The two constituents present in the core II population that are not well separated on the sucrose gradient, denoted core IIa and core IIb in Figure 1A to distinguish them from the mixed core II preparation, were purified from a nucleoprotein gel and individually characterized in some experiments reported below.

**RESULTS**

**The in vitro reconstitution of a 214 bp DNA fragment results in several distinct nucleosome core species**

A 214 bp Asp718–XbaI fragment (AX fragment) from the histone H1–H4 spacer region of the early histone gene battery of the sea urchin *P. miliaris* was 3′-labeled at one end and reconstituted into a nucleosome core by high salt-mediated exchange of the octamer from H1/H5-stripped chicken erythrocyte chromatin. The reconstituted AX fragment was further purified by centrifugation on an isokinetic sucrose gradient to remove the long chromatin histone source. This manipulation resolved the reconstituted AX fragment into three major populations, as shown in Figure 1A. The histone composition of each of the three populations was determined by a difference probability analysis as previously described (32,33).

**Difference probability analysis**

The difference in the accessibility of the DNA helix to DNase I in a reconstituted complex compared with the free DNA was determined by a difference probability analysis as previously described (32,33).

**suGF1 forms a stable ternary complex only with core I**

The ability of the purified suGF1 protein to stably bind to the oligo(dG-dC) recognition sequence in a reconstituted core was investigated by incubating suGF1 with purified core I, II and III and resolving the resulting nucleoprotein complexes on a native 4% polyacrylamide gel (note that where we refer to core II in the text, we imply a mixture of cores IIa and IIb). The result is shown in Figure 2A. The incubation of suGF1 with free DNA (lanes 2 and 3) results in the appearance of two bands with a reduced electrophoretic mobility. The complexes present in these two bands were previously shown to be structurally indistinguishable complexes of DNA-bound suGF1 (21) and may correspond to different post-translational modifications of suGF1 or the presence of a slightly truncated version of the protein in the purified preparation (21). Looking next at the interaction of suGF1 with core I (lane 5), a shifted band is obtained (indicated by the arrow in Fig. 2A) that is not aligned with any of the complexes formed between suGF1 and free DNA (lanes 2 and 3) and is not present in the core I preparation incubated in the absence of suGF1 (lane 4). Neither core II, which resolves as core IIa and core IIb on the gel (lane 7), nor core III (lane 9) produced a similarly shifted band following incubation with suGF1. This result shows that a stable ternary complex can be formed between suGF1 and core I, but not between suGF1 and core IIa, core IIb or core III. Note that there is no re-equilibrium among the purified core species (lanes 4, 6 and 8) under the incubation conditions employed for suGF1 binding. This result suggests that suGF1 is able to bind
to core I due to the unique, stable structure of this specific nucleosome core.

It was previously shown that incubation of the AX fragment with increasing amounts of suGF1 results in the progressive appearance of suGF1–DNA multimers (21). If the interaction of suGF1 with the nucleosomal DNA of core I is weak, it is possible that the molar excess of suGF1 over the small amount of dissociated free DNA in the core I preparation may result in the appearance of such suGF1–DNA multimers. To address the possibility that the band identified as the suGF1–core I triple complex (lane 5 of Fig. 2 A) in fact represents a suGF1–DNA multimer, both free AX fragment and core I was titrated with increasing amounts of suGF1 (Fig. 2B). The result clearly shows that the identified ternary complex of suGF1–core I is not aligned with any of the suGF1–DNA multimers, indicating that the complex indeed represents suGF1 bound to the nucleosomally wrapped AX fragment of core I. Note also that suGF1 preferentially binds to core I even if core IIb is present in the incubation mixture (Fig. 2B).

**The interaction of suGF1 with core I is sequence-specific**

The sequence specificity of the suGF1–core I interaction was investigated by competing for suGF1 with specific and non-specific double-stranded oligonucleotides. The result is shown in Figure 2C.

The presence of increasing amounts of unlabeled specific competitor DNA containing the suGF1 binding site in the incubation mixture results in a systematic decrease in the amount of suGF1–core I triple complex (compare lane 3 with 7). In the presence of a 10-fold molar excess of specific competitor DNA over nucleosomally wrapped AX fragment, formation of the ternary complex is effectively abolished (lane 6). In contrast, even in the presence of a 20-fold molar excess of non-specific competitor DNA, no decrease in the amount of suGF1–core I complex is detectable (lane 14). This result shows that the suGF1–core I ternary complex represents the sequence-specific binding of suGF1 to the nucleosomally wrapped AX fragment in core I.

In order to gain insight into the ability of suGF1 to sequence-specifically bind to its recognition sequence in the nucleosomally wrapped AX fragment of core I, but not that of core IIa, core IIb or core III, the nucleosomal structures of these core species were investigated by MNase, DNase I and hydroxyl radical footprinting.
radiolabeled at the XbaI end (Fig. 3). Two series of exo III pauses are visible at positions –444, –429, –420 and –410 (these bands are not readily visible in Fig. 3, which was exposed for optimal visualization of the smaller bands) and at positions –334, –344, –353 and –363, resulting in bands of 100, 110, 120 and 130 bp respectively. The asterisks labeled 5–8 indicate cuts at positions –350, –340, –330 and –320, resulting in bands of 101, 111, 120 and 130 bp respectively. The standards indicated in bp in the lanes labeled M are an HpaII digest of pBR322, radiolabeled by Klenow fill-in. The oligo(dG·dC) suGF1 binding site is identified by the brackets in the margins of the figure.

The rotational placement of the histone octamer in cores Ia+Ib, IIa, IIb and III

The rotational position of the histone octamer in the identified core species was investigated by DNase I and hydroxyl radical cleavage. The digested DNA was isolated from each core complex resolved on a polyacrylamide gel following DNase I digestion and the digested DNA isolated from the appropriately shifted complex on the gel. The purified DNA samples were electrophoresed on a 6% polyacrylamide–8 M urea gel. Identical samples were loaded on the same gel at different times to resolve the DNA digestion pattern at the XbaI (long) or Asp718 (short) end of the AX fragment. The sequence positions are given on either side of each panel and the binding site of suGF1 is identified by the bracket alongside each panel. Lane M shows Maxam–Gilbert G sequencing reaction products.

An interesting feature in the hydroxyl radical cleavage of the free AX fragment (Fig. 5, lane 1) is the clear periodicity with maxima at positions –277, –287, –297, –307 and –317. These maxima most likely correspond to regions where the minor groove is expanded (35) due to the previously identified curvature
Figure 5. Hydroxyl radical digestion of reconstituted nucleosome cores and suGF1 complexes. Free AX fragment $^{32}$P-labeled at the XbaI site (Watson) (lane 1), suGF1–DNA binary complex (lane 2), core I (lane 3), suGF1–core I ternary complex (lane 4), core IIa (lane 5), core IIb (lane 6) and core III (lane 7) were digested with hydroxyl radicals. All suGF1 and core complexes were resolved on a polyacrylamide nucleoprotein gel following hydroxyl radical digestion and the digested DNA isolated from the appropriately shifted complex on the gel. The DNA samples were analyzed as described in Figure 4.

of this sequence in solution (26). It is further noteworthy that the cleavage maxima in the free DNA coincide with that found in the corresponding region of all the core species, demonstrating that the direction of curvature of the free AX fragment is accommodated in the rotational orientation of the DNA duplex on the octamer surface.

The structural organization of the suGF1–core I ternary complex at single nucleotide resolution

The association of suGF1 with the AX fragment results in the protection of $\sim 20$ bp of DNA enclosing the oligo(dG·dC) motif from DNase I cleavage (Fig. 4 A and B, lanes 2), as previously reported (21). The DNase I digestion of the suGF1–core I ternary complex (Fig. 4A, lane 4) also shows this characteristic suGF1 footprint, indicated by the bracket in Figure 4. However, the DNase I cleavage periodicity seen between sequence positions $-277$ and $-339$ in core I (Fig. 4A, lane 3, the short run) is not easily detectable in the suGF1–core I triple complex (Fig. 4A, lane 4, the short run). In the ternary complex, the DNase I cleavage periodicity appears enhanced towards the Asp718 side of the AX fragment, most easily seen between sequence positions $-354$ and $-377$ on the labeled Crick strand (Fig. 4B, compare lanes 3 and 4, the short run). To formalize this visual impression, an analysis of the difference in the probability of DNase I cleavage in the DNA complexed with protein versus the free DNA was performed (32,33). The result is shown in Figure 6.

Figure 6. Difference probability plot of the DNase I digestion of core I and suGF1–core I ternary complex. Lanes 3 (core I) and 4 (ternary complex) of Figure 4 were densitometrically scanned and the differences in the probability of DNase I cleavage in the complexed DNA relative to the free DNA calculated for core I (A) and the suGF1–core I ternary complex (B). The difference probabilities, expressed as a natural logarithm, are shown in the top section of each panel for the Watson strand and in the bottom section for the Crick strand.

Core I (Fig. 6A) shows a clear 10 bp periodicity in the probability of cleavage in the core DNA between sequence positions $-277$ and $-339$, corresponding to the region that was found to be protected from MNase cleavage (data not shown). The weaker modulation at a similar period visible on both strands of the AX fragment between positions $-420$ and $-350$ is most likely due to the presence of core Ia in the core I preparation. In the case of the suGF1–core I ternary complex (Fig. 6B), this difference in the cleavage probability between the two halves of the complexed AX fragment is clearly reversed. No clear 10 bp periodicity is visible between sequence positions $-277$ and $-339$. In contrast, on the other side of the bound suGF1, towards the Asp718 end of the fragment, a clear 10 bp periodicity is visible between sequence positions $-408$ and $-354$. This result shows that the octamer is located on the Asp718 half of the DNA molecule in the ternary complex and demonstrates that suGF1 will preferentially bind to its recognition sequence specifically in core Ia.

DISCUSSION

We have shown that the in vitro reconstitution of a 214 bp fragment with the histone octamer results in the appearance of several structurally distinct nucleosome core species that contain the octamer in a similar or identical rotational frame, but in different translational settings on the DNA molecule. The various translational settings are summarized in Figure 7, which clearly shows that the placement of the suGF1 recognition sequence at a position where it is closely associated with the octamer surface essentially abolishes suGF1 binding. Referring to the DNase I accessibility of cores I, IIa and IIb (Fig. 4), it is seen that the major groove spanning the central G residues of the suGF1 binding site is orientated away from the octamer surface. However, the DNase I cleavage maximum within the DNase I footprint of the binary
will be present in a similar rotational orientation in a nucleosome in vivo (36). The finding that suGF1 does not bind to its binding site in cores Ila and IIb, the major translational frame on this DNA fragment, suggests that suGF1 must gain access to DNA immediately after DNA replication and prior to nucleosome deposition or that a complementary nucleosome disruptive activity is required in the normal in vivo function of this protein.

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Figure 7. Summary of the translational frames in the different nucleosome core species obtained with the in vitro reconstitution of the 214 bp AX fragment. The nucleosome cores (ellipses) are shown on the DNA molecule (solid line) in the translational frames assigned in the text. The sequence positions of the assigned nucleosome core borders are indicated above each schematic. The filled rectangle represents the suGF1 oligo (dG·dC) binding site. The individual core species are identified to the left of the figure. The assignment of the translational frames is based on the MNase digestion of cores I, II and III (data not shown) and on the exo III digestion of core I, discussed in the text. Note that the available data is insufficient to unambiguously assign core Ila and IIb to the two indicated core II species.