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Evidence for DNA Translocation by the ISWI Chromatin-Remodeling Enzyme

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The ISWI proteins form the catalytic core of a subset of ATP-dependent chromatin-remodeling activities. Here, we studied the interaction of the ISWI protein with nucleosomal substrates. We found that the ability of nucleic acids to bind and stimulate the ATPase activity of ISWI depends on length. We also found that ISWI is able to displace triplex-forming oligonucleotides efficiently when they are introduced at sites close to a nucleosome but successively less efficiently 30 to 60 bp from its edge. The ability of ISWI to direct triplex displacement was specifically impeded by the introduction of 5- or 10-bp gaps in the 3′-5′ strand between the triplex and the nucleosome. In combination, these observations suggest that ISWI is a 3′-5′-strand-specific, ATP-dependent DNA translocase that may be capable of forcing DNA over the surface of nucleosomes.

A general feature of eukaryotes is that their genomic DNA associates with proteins to form chromatin. In addition to providing a means of packaging DNA within nuclei, chromatin provides an additional level at which gene expression can be regulated. Active regions of the genome tend to be maintained in a more accessible state than inactive regions, and the manipulation of chromatin structure has been found to play an important role in the regulation of many genes. In order to be able to regulate gene expression at this level, eukaryotes have devised a number of strategies by which they can manipulate chromatin structure. These include the covalent modification of chromatin structure by posttranslational modification, the manipulation of the protein content of chromatin through the association of variant histone and nonhistone proteins, and the noncovalent alteration of chromatin structure by ATP-dependent chromatin-remodeling activities (2, 7, 38, 67).

It now appears likely that all eukaryotes encode multiple yet distinct ATPases with homology to the yeast SNF2 protein (Snf2p). These include the Sth1p, Chd1p, Isw1p, Isw2p, and Ino80p proteins in budding yeast and a spectrum of related proteins in higher eukaryotes (2, 66). Among these, the ISWI proteins represent a discrete class of ATPase involved in chromatin remodeling. The founding member of the ISWI group was identified in *Drosophila melanogaster* due to its similarity to Snf2p. As the homology is restricted to the helicase-like domain, it was named imitation SWI/SNF (ISWI) (21). Biochemical characterization of ISWI proteins began with the identification of ISWI as the catalytic subunit of three distinct complexes, nucleosome-remodeling factor (NuRF), chromatin accessibility complex (ChrAC), and the ATP-utilizing chromatin assembly and modifying factor (ACF) isolated from *Drosophila* embryo extracts (36, 60, 64). Subsequently, ISWI-related proteins have been found to be components of chromatin-remodeling complexes in organisms ranging from yeast to humans, suggesting that these proteins fulfill important functions conserved throughout evolution (5, 42, 43, 49, 61). Within each of these complexes, the ISWI protein is associated with a functionally conserved complement of additional proteins (2).

The biochemical fractionation of ISWI complexes has enabled their activity to be assessed by a variety of different assays. The ATPase activity of ISWI-containing complexes has been found to require nucleosomal histones for maximal stimulation (1, 12, 29). More recent studies have defined residues 16 to 19 of histone H4 as playing a critical role in the stimulation of ATPase activity (13, 33). Partial stimulation has been observed in the presence of complexes between the tail peptide and DNA (13). Thus, the ISWI ATPase is likely to recognize an epitope that includes H4 residues 16 to 19 within the context of a nucleosome. Recent studies have revealed that ISWI-driven ATP hydrolysis is capable of repositioning nucleosomes along DNA in *cis* (31, 34, 41). These observations, together with the finding that the dISWI protein alone is also able to carry out this reaction (41), suggest that nucleosome mobilization may be a common property of ISWI-containing complexes. This is an attractive proposition, as the ability to alter nucleosome positioning provides these complexes with a means to both organize nucleosomes into precisely spaced arrays (36, 64) and generate nuclease sensitivity within chromatin fibers (60, 64).

The ISWI proteins, like other ATP-dependent remodeling activities, contain seven motifs that are present in many helicase and helicase-like proteins (20). Recently, the high-resolution structures of a number of helicases have been determined. These include PcrA, which was shown to contain a tandem repeat of RecA-like domains (57). The structures of PcrA in two different DNA complexes show that the binding and hydrolysis of Mg^{2+} ATP results in a massive change in the rela-

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tive orientation of the two RecA-like domains (65), which could drive the enzyme along the DNA backbone. This translocase activity can be physically uncoupled from the strand separation activity of the enzyme (54), raising the possibility that many of the helicase-like proteins that do not function in strand displacement assays could in fact be ATP-dependent duplex DNA translocases. Consistent with this, the structure of RecG, a member of the superfamily II helicase-like proteins (SF2) to which ATP-dependent remodeling enzymes are most closely related, also suggests that helicase motifs can function as part of a duplex DNA translocase domain (53).

Other members of the SF2 family which do not have a strand-separating activity also appear to be DNA translocases. An assortment of assays have been used to obtain evidence that the DNA translocating subunits of type I restriction enzymes can translocate along DNA (18, 22, 23, 27, 37, 48, 59, 63). Also of particular interest is recent evidence suggesting that the yeast Mot1 and Rad54 proteins, which are more closely related to the ATP-dependent chromatin-remodeling enzymes, are able to translocate along DNA (16, 50). This makes the concept that ATP-dependent chromatin-remodeling activities may also be able to translocate along DNA attractive. Supporting this, several ATP-dependent remodeling activities have been shown to generate unconstrained superhelical torsion within linear DNA or chromatin fragments, which was proposed to occur as a result of the translocation of remodeling enzymes along the helical DNA backbone (35). More recent studies of the yeast RSC and *Drosophila* ACF complexes support the idea that these complexes also translocate along DNA (see Discussion) (26, 51).

Here, we adapted a triplex oligonucleotide displacement assay (23) to study the action of ISWI on nucleosomal substrates. This provides new evidence to support the idea that ISWI is capable of distorting the DNA adjacent to nucleosomes. We also found that the ATPase activity of ISWI is stimulated to a low yet detectable level in the presence of single-stranded DNA. This suggests that ISWI is capable of functioning on one DNA strand. Consistent with this, the displacement of triplex DNAs was blocked with specific polarity by gaps in the 3′-5′ DNA strand. As these gaps were positioned between the triplex and the nucleosome, the results support the theory that ISWI functions as a nucleosome-stimulated DNA translocase oriented such that it forces DNA over the surface of nucleosomes.

MATERIALS AND METHODS

Purification of proteins. Recombinant *Drosophila* ISWI and histones were purified as described previously by Corona et al. (14) and Luger et al. (44), respectively.

Preparation of template DNA. PCR-generated DNA was purified from oligonucleotide primers by fractionation over a 1-ml Source 15Q anion exchange column (Pharmacia). DNA fragments containing gaps and nicks were prepared by multiple-round primer extension as described previously (11), except that the resulting single-stranded DNA was purified by anion exchange chromatography. Templates were then prepared by annealing complementary single-stranded DNA in annealing buffer (10 mM Tris-Cl [pH 8.0], 50 mM NaCl). Further information about the DNA sequences used can be found at http://www.dundee.ac.uk/biocentre/owenhughes/MCB2003suppl.pdf.

TFO annealing. A total of 10 pmol of ^{32}P-radiolabeled TFO, TTCTTTTCTT TCTTCTTTCTTT, was incubated with 100 pmol of duplex DNA containing the triplex binding site in a 20-μl reaction volume containing 40 mM morpholineethanesulfonic acid (pH 5.5) and 10 mM $MgCl_2$. The mixture was heated to 57°C for 10 min and allowed to cool slowly to room temperature. These sequences formed a parallel triplex, meaning there were no complementary sequences capable of annealing with the TFO to form a heteroduplex.

Reconstitution of nucleosomes. Standard reconstitution reactions were conducted with equimolar amounts (100 pmol) of duplex DNA and purified *Xenopus* histone octamer as described previously (44). Low-pH TFO reconstitutions were conducted by mixing 100 pmol of TFO-bound DNA with 100 pmol of recombinant *Xenopus* histone octamer in a 50-μl reaction containing 1.2 M NaCl, 40 mM Na-cacodylate (pH 6.0), and 5 mM $MgCl_2$. This mixture was left to equilibrate at room temperature for 30 min and then placed in a microdialysis block containing Spectra/Por 1 dialysis tubing (cutoff, 11,000 to 15,000 Da; Spectrum). Stepwise dialysis was conducted at 4°C at 2-h intervals against buffer 0.85 CAM (850 mM NaCl, 20 mM Na-cacodylate [pH 6.0], 5 mM $MgCl_2$) and then 0.65 CAM (650 mM NaCl, 20 mM Na-cacodylate [pH 6.0], 5 mM $MgCl_2$), followed by 0.5 CAM (500 mM NaCl, 20 mM Na-cacodylate [pH 6.0], 5 mM $MgCl_2$) and finally 0.1 CAM (100 mM NaCl, 20 mM Na-cacodylate [pH 6.0], 5 mM $MgCl_2$). The level and homogeneity of reconstitutions were assessed by native gel electrophoresis. Reconstitutions were then stored at 4°C.

Gel shift assays. DNA and nucleosome binding were assayed by electrophoresis through 5% acrylamide–0.5× Tris-borate-EDTA (TBE) gels with 1× TBE as running buffer. Gels were run either in the cold room or at room temperature at 70 to 100 V as indicated in the figure legends. Binding reactions contained 3 mM $MgCl_2$, 20 mM HEPES (pH 7.9), 400 μg of bovine serum albumin (BSA)/ml, 0.033% NP-40, 1 mM dithiothreitol, 5% glycerol or 2.5% Ficoll, and 60 or 120 mM NaCl as indicated in the figure legends.

ATPase assays. ATPase reactions were carried out in the presence of 20 mM HEPES (pH 7.9)–3 mM $MgCl_2$–0.033% NP-40–20% glycerol–1 mM dithiothreitol–400 μg of BSA/ml–5 or 10 μM ATP–2.2 μCi [α^{32}P]ATP and differing amounts of nucleosomes, DNA, NaCl, and ISWI as indicated in the figure legends. Reactions were incubated at 30°C, and at the indicated time points, 1/10-volume aliquots were removed from the 10- or 20-μl reactions and stopped by addition to 10% sodium dodecyl sulfate. A total of 1 μl of each stopped reaction was analyzed by thin-layer chromatography (TLC) on polyethylenimine cellulose F TLC plastic sheets (Merck) with 0.75 M KH_2PO_4 (pH 3.5) as the running buffer to separate ATP from ADP. The extent of hydrolysis was determined by quantification of the spots corresponding to ATP and ADP with a PhosphorImager (Molecular Dynamics).

Site-directed mapping. High-resolution nucleosome mapping was conducted as described previously (25). A total of 2 pmol of each ^{32}P-end-labeled substrate was incubated for 30 min at 30°C in a 20-μl reaction containing 0.25 pmol of dISWI protein in the presence or absence of 1 mM Mg^{2+} ATP. The reaction was stopped by the addition of an excess of lambda DNA competitor and chilled on ice. Site-directed cleavage was initiated by the addition of 15 μM $(NH_4)_2Fe(SO_4)_2$, followed by 3 mM ascorbic acid and 0.05% H_2O_2 for 45 min. Reactions were stopped by the addition of 1 M thiourea and 10 mM EDTA. Samples were precipitated and analyzed by denaturing gel electrophoresis.

TFO displacement assays. TFO displacement reactions were conducted in 20-μl reactions in 20 mM Na-HEPES (pH 7.9)–50 mM NaCl–3 mM $MgCl_2$–10 μg of BSA (Promega)/ml–5% glycerol. All reactions contained 500 fmol of TFO-bound mononucleosomes. Mg^{2+} ATP or Mg^{2+} ATPγS was added to a final concentration of 1 mM. dISWI was added to a final concentration of 12.5 nM (250 fmol). All reactions were conducted for 30 min at 30°C. Displacement was assayed on a 1.5-mm-thick 5% (wt/wt) polyacrylamide gel containing 0.25× TAM (40 mM Tris-acetate [pH 7.0], 1 mM $MgCl_2$). Electrophoresis was performed with 1× TAM buffer, after which the presence of the TFO was monitored with a PhosphorImager.

RESULTS

Binding of ISWI to nucleosomes and DNA. Previously, it was reported that the ISWI protein preferentially interacts with nucleosomes containing linker DNA (6). In order to investigate this further, the ability of the ISWI protein to bind to nucleosomal substrates containing defined lengths of linker DNA was studied. A prerequisite for this is the ability to position nucleosomes precisely. In order to achieve this, the well-characterized nucleosome A (NucA)-positioning sequence found in the mouse mammary tumor virus (MMTV) long terminal repeat was used (25). We adopted a nomenclature in which the DNA fragments are described by the term

xAy, where x denotes the length of DNA attached to the upstream side of the nucleosome, A denotes the 147-bp positioning sequence, and y denotes the length of linker DNA attached on the downstream side of the nucleosome. For the nucleosome binding studies described here, we added linker DNA to one side of the nucleosome. The reason for this was that nucleosomes can be reconstituted to the end positions with a greater homogeneity than at central locations (data not shown). Figure 1A shows the binding of ISWI to 0A0 nucleosome core particles and 0A41-bp nucleosomal fragments. As reported previously, ISWI was found to form complexes stable enough to be resolved as discrete species during native gel electrophoresis with nucleosomes containing linker DNA but not with nucleosome core particles (6). However, we also noted that when the disappearance of free nucleosome was compared, there was little difference between the substrates. Thus, ISWI is capable of interacting with nucleosome core particles but discrete complexes are not formed.

We also noticed that the low levels of free DNA present in our nucleosome reconstitutions were shifted in the presence of ISWI. This suggests that ISWI has the ability to bind to free DNA. In order to investigate this further, we measured the binding of ISWI to DNA fragments of different lengths (Fig. 1B). Although we could not detect ISWI binding to a 15-bp DNA fragment, it was able to bind to DNA fragments of 23, 32, and 41 bp with increasing efficiency. To characterize the length dependence of ISWI binding to DNA more carefully, ISWI binding was monitored over a range of DNA concentrations for fragments of 32, 41, and 77 bp. The results plotted in Fig. 1C illustrate that, whereas the 41-bp fragment interacts with ISWI more efficiently than the 32-bp fragment, the 77-bp fragment does not further enhance ISWI binding. This suggests that the minimal DNA length for interaction with ISWI is between 15 and 23 bp and that a 41-bp fragment is capable of optimally interacting with ISWI.

The stable complexes formed during the binding of ISWI to the 41-bp fragment are similar to those observed during binding to nucleosomal DNA. This raises the possibility that the interaction of ISWI with DNA may contribute to the previously observed preference of ISWI for nucleosomes containing linker DNA (6, 40). Indeed, the nonspecific DNA binding properties of ISWI might be expected to bias the interaction towards linker DNA or relatively accessible DNA at the edge of the nucleosomes. This complicates the interpretation of the location of ISWI in bulk ISWI-nucleosome assemblies, as the most stable complexes may not be those in which the ISWI has engaged with the nucleosome in a functional orientation. For example, the functional interaction of ISWI might be transient and perhaps related to the smearing and aggregation we observed during the binding of ISWI to the 0A0 and 0A41 nucleosome core particles. In this case, the stable-shifted complexes observed with 0A41 nucleosomes could result from the nonfunctional binding of ISWI to the DNA adjacent to the nucleosome. Consistent with this, the presence of the H4 tail, which is critical for ISWI function, does not affect the ability of ISWI to gel shift nucleosomal DNA (12).

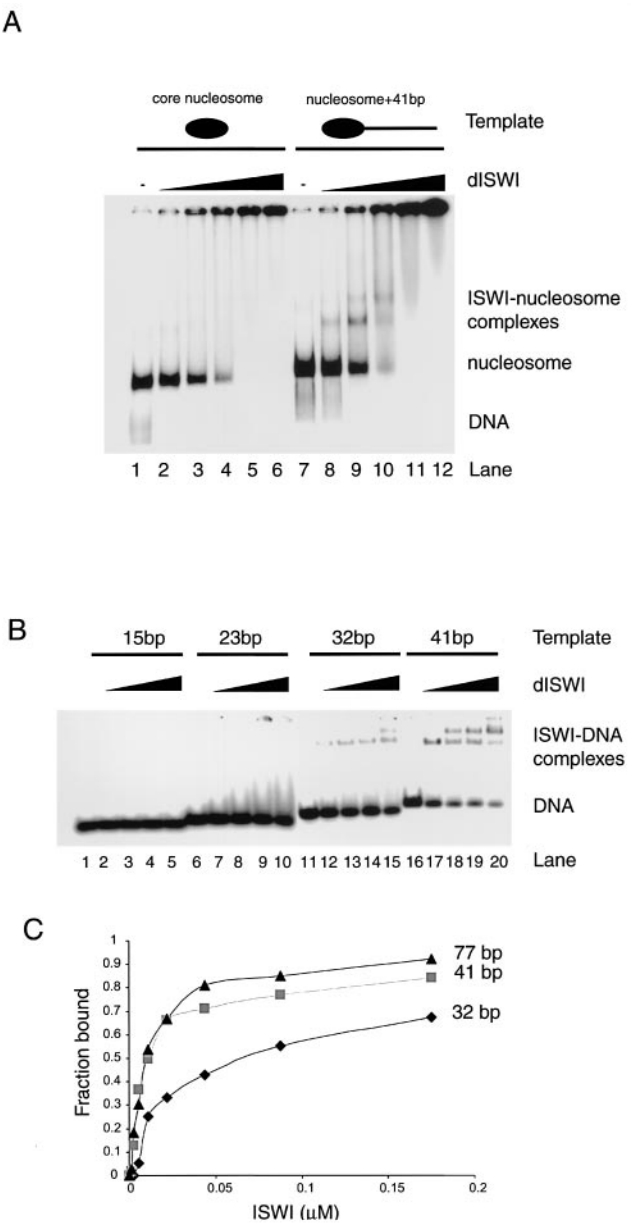

FIG. 1. Binding of ISWI to nucleosomal and free DNA. (A) Gel shift assay of ISWI binding to nucleosomes with no overhanging DNA and 41 bp of extra DNA. Binding reactions contained 5% glycerol, 60 mM NaCl, and 0.5 nM nucleosomes. ISWI was present at 0 pmol (lanes 1 and 7), 0.125 pmol (lanes 2 and 8), 0.25 pmol (lanes 3 and 9), 0.5 pmol (lanes 4 and 10), 1 pmol (lanes 5 and 11), and 2 pmol (lanes 6 and 12). (B) Gel shift assays showing the binding of ISWI to DNA fragments of increasing length. Binding reactions contained 2.5% Ficoll, 120 mM NaCl, and 0.5 nM DNA. ISWI was incubated with twofold increments of ISWI ranging from 0.125 to 2 pmol as described for panel A. (C) Plot illustrating the binding of ISWI to 31-, 41-, and 77-bp DNA fragments. The fraction bound was calculated from the disappearance of free DNA.

DNA length affects ISWI ATPase activity. In order to gain further insight into ISWI function, we next determined the ability of nucleosomal substrates of different DNA lengths to function as substrates for ISWI-driven ATP hydrolysis. Figure 2 shows that, whereas nucleosome core particles produce low levels of ATPase activity, nucleosomes containing successively longer lengths of DNA stimulate progressively higher levels of activity. We also found that nucleosomes with linker DNA 61 bp in length did not further stimulate ISWI ATPase activity

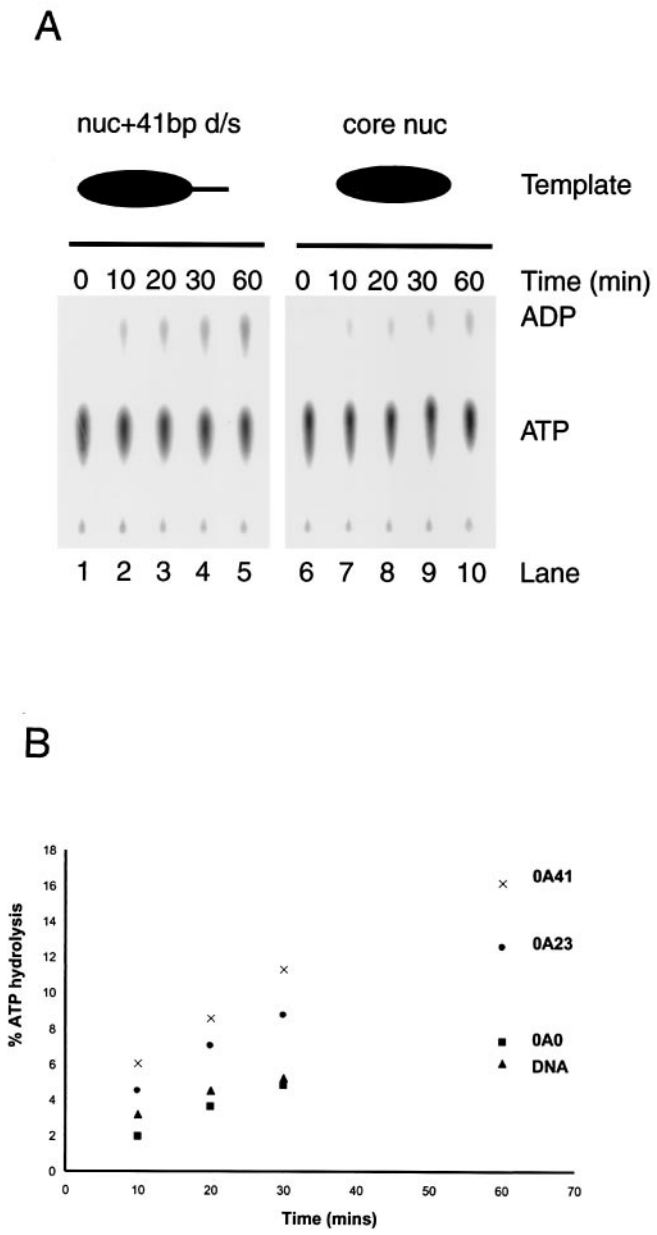

FIG. 2. Maximal stimulation of ISWI ATPase activity requires DNA outside the nucleosome. (A) TLC plate showing a time course of ATP hydrolysis with a nucleosome core and a nucleosome core with 41 bp of linker DNA. Reactions (20 μl) contained 11 pmol of ISWI, 9.5 pmol of nucleosomes, 100 mM NaCl, and 5 μM ATP. (B) Time course of ATP hydrolysis by ISWI in the presence of 9.5 pmol of nucleosomes with increasing sizes of linker DNA. 0A0 is a 147-bp nucleosome core derived from the MMTV NucA-positioning sequence, 0A23 is the same sequence with 23 bp of linker DNA attached to one side, and 0A41 has 41 bp of DNA attached to one side. DNA is a 208-bp fragment encompassing the NucA-positioning sequence.

(data not shown). This implies that ATP hydrolysis involves the interaction of ISWI with DNA outside of the nucleosome core.

DNA fragments have also been reported to be capable of stimulating low levels of ISWI ATPase activity (14). Therefore, we next compared the ability of DNA fragments of increasing length to stimulate the ATPase activity of ISWI. Although the efficiencies of stimulation were lower than those observed with nucleosomal DNA, Fig. 3 illustrates that DNA length does have an effect per se. While a 15-bp fragment was capable of only modest levels of stimulation above the background levels observed in the absence of DNA, 23-, 32-, and 41-bp fragments generated successively higher levels of stimulation. However, increasing the length of the DNA beyond 41 bp resulted in an only modest additional stimulation of ATPase activity.

Previously, it was reported that the ATPase activity of the SWI/SNF and RSC complexes can be stimulated by single-stranded nucleic acids (8, 9). For this reason, we next investigated whether single-stranded nucleic acids were also capable of stimulating the ATPase activity of the ISWI. We found that the stimulation of ISWI ATPase activity by single-stranded DNA was less efficient than that when using double-stranded DNA; however, by using higher concentrations of ISWI, this stimulation could be detected (Fig. 3A and C). We next investigated whether the stimulation of ISWI ATPase activity by single-stranded DNA was dependent on the fragment's length. Figure 3A and C illustrates that the ability of single-stranded DNA to stimulate the ATPase activity of ISWI displays a length dependence similar to that observed for double-stranded DNA fragments, with maximal stimulation occurring on fragments of approximately 40 bp.

The dependence of ISWI ATPase activity upon DNA length both within the context of a nucleosome and on free DNA suggests that DNA contacts over a region of ~40 bp are required for maximal stimulation. One possible explanation for this DNA length dependence is that ATP hydrolysis by the ISWI protein involves DNA translocation and that the shorter length of some of the templates we used reduces the length of DNA over which translocation is possible. An alternative explanation is that ISWI must contact DNA with a length of approximately 40 bp in order to fully activate its ATPase activity. Ideally, the next step would be to determine whether these contacts primarily affect the K_m or V_{max} of the reaction. Unfortunately, the activity of ISWI in ATPase assays is low, meaning that we were not able to perform ATPase assays over a broad enough range of concentrations and time points to accurately determine these kinetic parameters. Instead, we developed a new approach to monitor the action of the ISWI protein.

ISWI-dependent displacement of a TFO. The DNA translocating subunits of type I restriction enzymes are, like the ATP-dependent remodeling activities, members of the SF2 family of helicase-like proteins. Recently, an assay was developed to measure the motion of the type I restriction enzyme *Eco*R124I along DNA (23). Briefly, this assay involves the annealing of a pyrimidine-rich triple-helix-forming oligonucleotide (TFO) to a purine-rich target sequence. The pyrimidine-rich TFO occupies the major groove parallel to the purine-rich strand and utilizes Hoogsteen hydrogen bonds to form T-AT and C^+-GC triplets (30). The formation of the triple helix depends upon N3 cytosine protonation and is dependent on low pH; the triplex does not form at a neutral pH. However, this reliance on low pH can be circumvented by preforming the triplex at a low pH and then diluting it into reaction buffer and by the inclusion of millimolar concentrations of divalent cations, which stabilize the triplex indefinitely (39). Importantly, at a physiological pH, the TFO, once displaced, is unable to rebind.

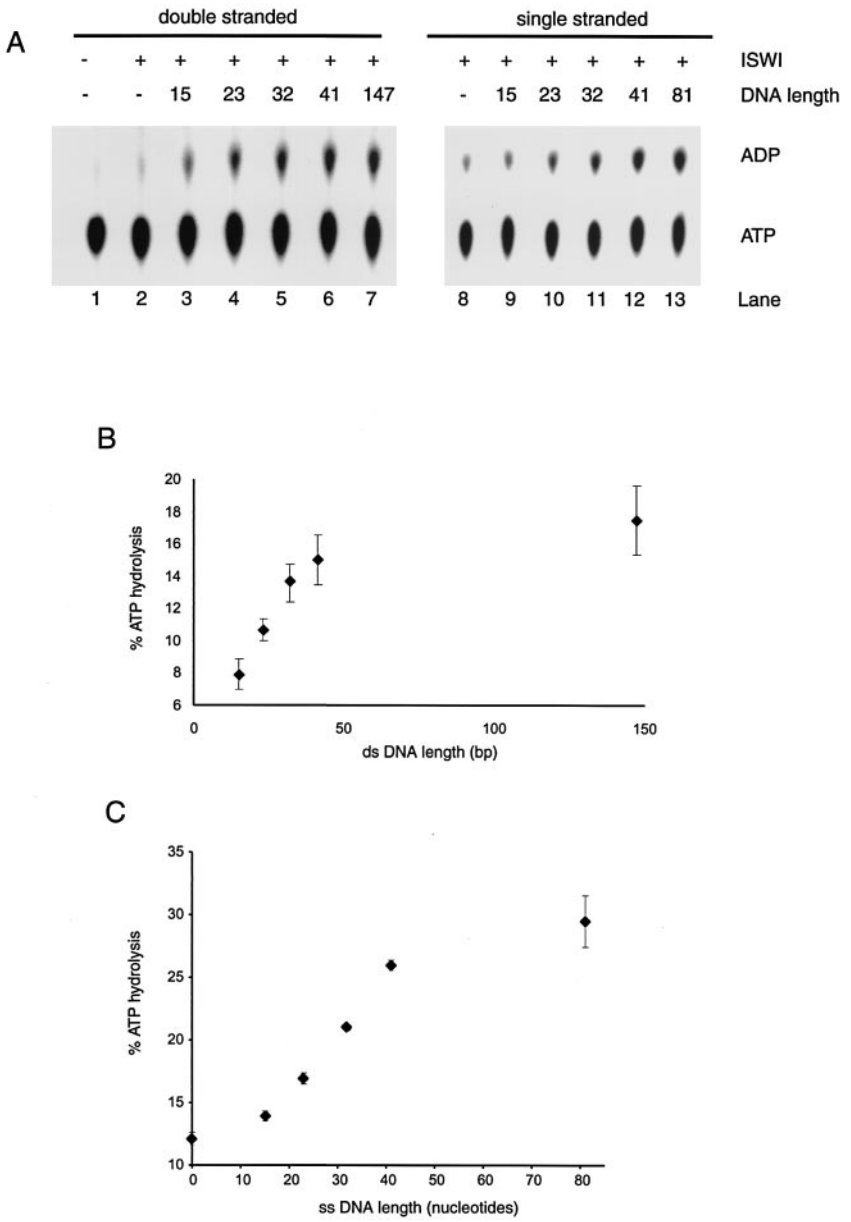

FIG. 3. ISWI ATPase activity is stimulated by double- and single-stranded DNA in a length-dependent fashion. (A) Resolution of the products of typical ATPase reactions by TLC; (B and C) levels of ATP hydrolysis following stimulation with double- and single-stranded DNA, respectively. Reactions (10 μl) contained 40 pmol of duplex DNA, 10 μM ATP, and 200 mM NaCl. As lower levels of ATP hydrolysis were obtained in the presence of single-stranded DNA (ss DNA), high levels of ISWI (66 pmol) were included in these reactions compared to those in the reactions with double-stranded DNA (ds DNA) (12 pmol). The data points plotted represent the average ATP hydrolysis after 1 h at 30°C for three separate reactions. Further details are given in Materials and Methods.

As a result, triplex displacement provides a means of monitoring the action of DNA translocases that are capable of displacing the TFO (32).

We have adapted this assay to study the action of ISWI. In order to do this, we first studied the displacement of TFOs from sites adjacent to centrally located nucleosomes. Although triplex displacement did occur, nucleosomes were also repositioned on these templates, making it difficult to distinguish whether ISWI or the movement of the nucleosome had caused the triplex to be displaced (data not shown). In order to study the action of ISWI in triplex displacement, we sought a means to distinguish between direct and indirect displacement. With this aim, we exploited the fact that ISWI has previously been reported to move mononucleosomes from central positions to the end position but not visa versa (41). The MMTV NucA-positioning sequence was used to generate nucleosomes that were positioned either centrally (61A61) or at the upstream end of the DNA fragments (0A75). To confirm whether nucleosomes were assembled at the expected locations, we used high-resolution nucleosome mapping (24). Briefly, this involves the tethering of cysteaminyl EDTA to a recombinant histone octamer via a thiol group introduced at H4 cysteine 47.

The chelation of Fe^{2+} ions by the mapping reagent is capable of catalyzing the Fenton reaction, which results in the generation of hydroxyl radicals that are capable of cleaving DNA, but only within a range of approximately 1 nm. The site at which the reagent is attached on histone H4 comes into close contact with DNA approximately 4 bp on either side of the nucleosome dyad, meaning that the sites of cleavage can be used to deduce nucleosome positions. In the case of the 61A61 and 0A75 fragments, the major sites of cleavage prior to remodeling indicate that nucleosomes were predominantly located to the expected sites (Fig. 4A, lanes 1 and 3). Mononucleosome templates were prepared that contained the derivatized histone octamer either in the middle or at the upstream end of the DNA fragment (Fig. 4A).

Following ISWI remodeling on the 61A61 fragment, much of the cleavage at the major initial location of the nucleosome was lost and new cleavage sites were observed that are consistent with the redistribution of this nucleosome to positions located close to either end of the DNA fragment (Fig. 4A, lane 2). However, remodeling of the terminally located nucleosome (0A75) under the same conditions did not result in a similar reduction in cleavage at the initial location, suggesting that ISWI is not able to relocate a significant proportion of the nucleosomes assembled on this template (Fig. 4A, lane 4).

Having established that the majority of the histone octamers residing at this terminal location are not relocated by dISWI, the ability of the enzyme to facilitate TFO displacement was investigated. Seven constructs were generated with a sequence capable of annealing to the TFO 0, 10, 20, 30, 40, 50, or 60 bp downstream from an end-positioned nucleosome. Radiolableled TFO was annealed to these DNAs, and they were then assembled into nucleosomes. During native gel electrophoresis in TAM buffer (see Materials and Methods), TFO remained associated with the DNA, enabling species with the mobility of the TFO-bound nucleosome and TFO-bound free DNA to be resolved (Fig. 4B). Under these conditions, the free TFO migrated with the solvent front and was not shown. Restriction enzyme digestion was used to confirm that nucleosomes assembled onto the MMTV NucA-positioning sequence as expected when TFO was bound to the linker DNA (data not shown).

Incubation with dISWI and ATP resulted in efficient TFO displacement from constructs containing triplex DNA 0, 10, or 20 bp from the nucleosome. This was shown by a loss in intensity of the band corresponding to nucleosomal DNA (Fig. 4B, lanes 2, 4, and 6). Note that TFO was not removed from free DNA as would be expected given that the ISWI ATPase activity was maximally activated by nucleosomal histones. However, when displacement was assessed on constructs 0A54(tfo30) and 0A54(tfo40), where the TFO begins either 30 or 40 bp away from the nucleosome, the displacement was reduced (Fig. 4B, lanes 8 and 10). When triplex DNA was located 50 or 60 bp from the downstream edge of the nucleosome, no displacement was observed (lanes 12 and 14).

Figure 4C shows a plot illustrating the levels of ISWI-driven triplex displacement at each site. The fact that displacement becomes progressively less efficient further away from the edge of the nucleosome would be consistent with it occurring as a result of the DNA translocase activity originating from within or close to the edge of the nucleosome and oriented away from it. This would result in a collision between the ISWI and the triplex and, eventually, the displacement of the TFO. Limited processivity of this translocase could explain why displacement is more efficient close to the nucleosome than it is from further away. However, it also remains possible that some other form of DNA distortion could be responsible for triplex displacement.

Triplex displacement is impeded by strand-specific DNA lesions. If triplex displacement occurred as a result of ISWI translocase activity originating from within or close to the edge of the nucleosome, then insertion of lesions between the nucleosome and the TFO might be expected to interfere with triplex displacement. In order to test this, we generated a template in which a series of nicks, 5- or 10-bp gaps, were introduced into either DNA strand between the triplex and the nucleosome.

As expected, when supplemented with hydrolysable ATP, a control construct that contained no modification was able to facilitate dISWI-mediated TFO displacement (Fig. 5, lane 2). In a similar manner, both constructs containing nicks in either strand and 5- or 10-bp gaps in the top (5′-3′) strand showed normal displacement profiles (lanes 4 and 10). However, insertion of a 5- or 10-bp gap at the same position in the bottom (3′-5′) DNA strand was unable to support TFO displacement (lanes 12 and 14). This supports the involvement of one-dimensional DNA translocation in triplex displacement, as other forms of DNA distortion would not be expected to show this strand-specific polarity. The fact that gaps between the triplex and the nucleosome prevent displacement provides further evidence that translocation is oriented away from the nucleosome. Moreover, the observation that a gap in the 5′-3′ strand supports displacement suggests that translocation is occurring predominantly along the 3′-5′ DNA strand.

DISCUSSION

ISWI proteins form the catalytic core of a range of chromatin-remodeling activities that play important roles in gene regulation. In this study, we have used a range of different approaches to define the interaction between the ISWI ATPase and nucleosomal substrates. As reported previously, we found that ISWI forms more stable complexes with nucleosomes that contain additional linker DNA (6). However, we also found that ISWI binds to DNA fragments in a length-dependent fashion. Binding to a 23-bp fragment was weak and undetectable on a 15-bp fragment. ISWI bound to 32- and 41-bp fragments with greater affinity, and binding to these longer fragments was observed to result in the formation of more discrete complexes. We next investigated the dependence of ISWI ATPase activity on nucleosomal linker DNA length and found that this also displays length dependence. Although we found that ISWI ATPase activity is stimulated to a lesser extent in the presence of free DNA as reported previously, we also found that this stimulation showed a dependence on DNA length that peaked at approximately 40 bp. The ATPase activities of both SWI/SNF and RSC have previously been reported to be stimulated by both double- and single-stranded DNAs (9, 15, 51). Here, we report that the ATPase activity of ISWI is also stimulated by single-stranded DNA. In addition, we found that the stimulation of ISWI ATPase activity by single-stranded DNA

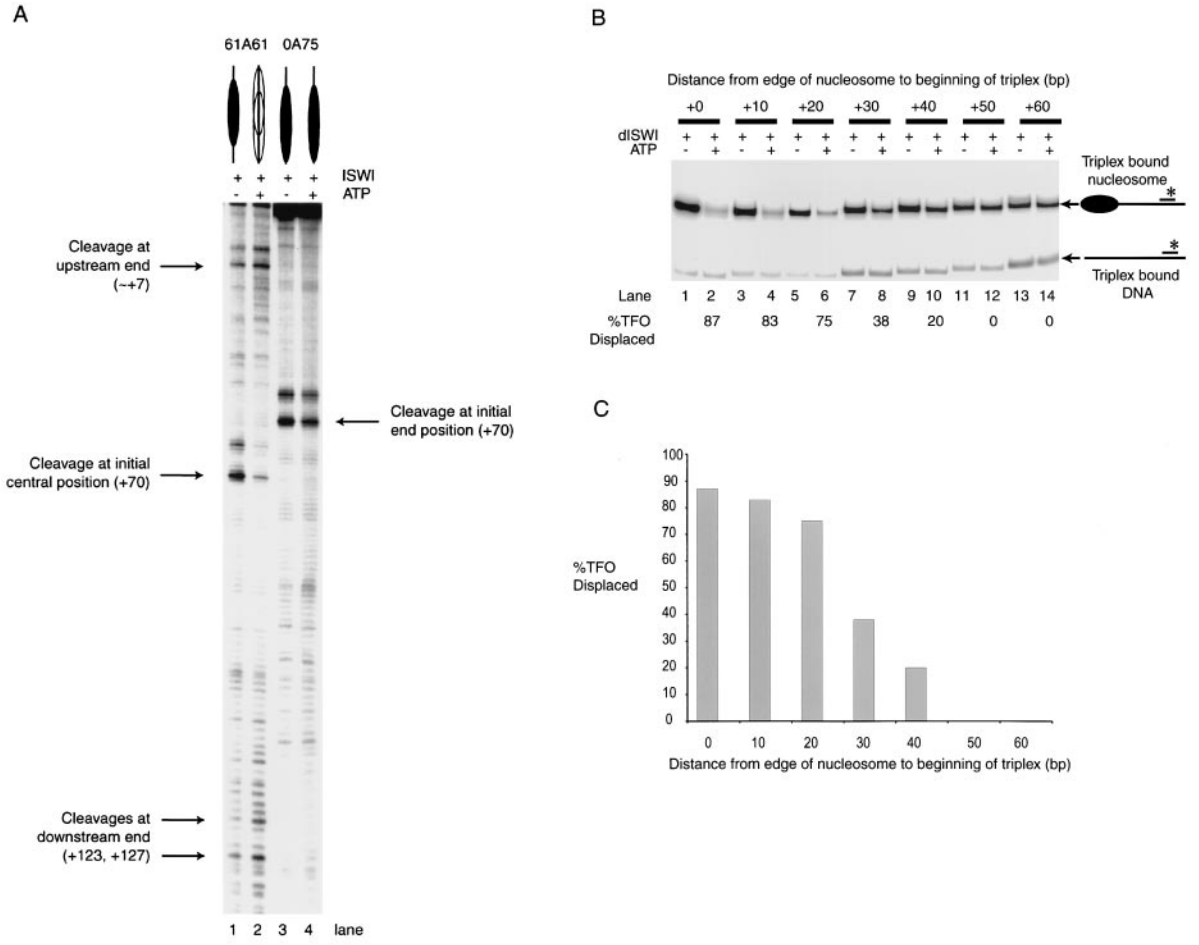

FIG. 4. ISWI-driven triplex displacement in the absence of nucleosome movement. (A) High-resolution site-directed nucleosome mapping was used to determine that an end-positioned nucleosome was not relocated by the action of the dISWI protein. Nucleosomes that were either centrally located on the DNA fragment 61A61 (lanes 1 and 2) or end positioned on the fragment 0A75 (lanes 3 and 4) were subjected to site-directed nucleosome mapping following incubation with ISWI in the presence or absence of ATP as indicated. The centrally located nucleosome was relocated to either end of the DNA fragment following remodeling, but the end-positioned nucleosome was not significantly repositioned. ISWI was also unable to alter the position of end-positioned nucleosomes when the triplex binding sequences were inserted in the adjacent DNA (data not shown). (B) A series of constructs were prepared that contained an end-positioned histone octamer and a 22-nucleotide, P^{32}-labeled TFO positioned at 10-bp intervals, progressively more distant to the edge of the nucleosome. Each template was incubated with dISWI in the presence or absence of ATP. (C) Graph illustrating that the level of TFO displacement decreased as the triplex DNA was positioned further from the nucleosome.

shows a length dependence that correlates well with that observed using duplexes. The DNA length dependence that ISWI displayed in these assays raises the possibility that ISWI functions as an ATP-dependent DNA translocase.

In order to test this further, we adapted a triplex displacement assay to study the action of ISWI on nucleosomal templates. ISWI was able to displace TFOs most efficiently from sites close to the edge of the nucleosomes. However, this activity reduced to undetectable levels 50 bp from the edge of a nucleosome. This would be consistent with ISWI, exhibiting a DNA translocase activity oriented away from the edge of nucleosomes with limited processivity. Further evidence supporting the orientation of the translocase activity was obtained through the observation that the introduction of gaps between the nucleosome and the triplex could prevent triplex displacement in a strand-polarity-dependent manner.

While this work was in preparation, two reports were published presenting evidence that chromatin-remodeling activities translocate along DNA (26, 51). Fyodorov and Kadonaga used a template commitment assay to show that the ISWI-containing ACF complex remains associated with, and preferentially functions on, one template following exposure to an equivalent amount of a competing template. This commitment to the template to which ACF is exposed first depends upon ongoing chromatin assembly and may offer valuable new insight into the way in which ACF functions. Fyodorov and Kadonaga argue that the phenomenon of template commitment provides evidence that ACF functions as an ATP-dependent DNA-translocating motor. However, template commitment has been observed previously for many DNA and chromatin binding proteins that are not ATP-dependent DNA translocases (3, 46). In order to explain template commitment, it has been proposed that, during transient dissociation from a site on one template, a DNA binding protein will be more

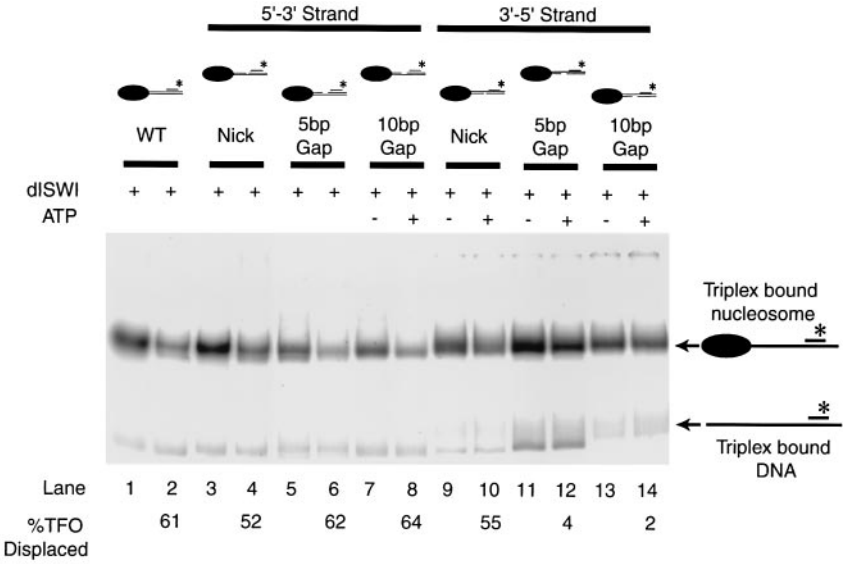

FIG. 5. Strand-specific DNA lesions prevent ISWI-driven triplex displacement. DNA templates were prepared that contained nicks, 5- or 10-bp single-stranded gaps, in either of the two DNA strands. 5′-3′, the top DNA strand; 3′-5′, the bottom DNA strand (with the nucleosome placed on the left). Each of the DNA lesions began 5 bp from the edge of the nucleosome; in addition, each template contained a ^{32}P-labeled TFO positioned 20 bp from the edge of the nucleosome. Each template was incubated with dISWI in the presence or absence of ATP for 30 min at 30°C.

likely to reassociate with the same template, as the local concentration of the molecule to which it was originally bound will be far higher than that of other molecules in the solution (55). It is difficult to rule out the possibility that similar processes explain the observations made by Fyodorov and Kadonaga.

The study by Saha et al. investigated the DNA length dependence of ATP hydrolysis by the yeast RSC complex and its catalytic subunit Sth1p. Both of these activities are more potent in ATP hydrolysis than ISWI. This has enabled a more thorough investigation of the relationship between the DNA length and ATPase activity to be performed than has been possible with ISWI. Importantly, for both Sth1 and RSC, it has been possible to establish that DNA length affects ATP hydrolysis by affecting the maximal rate of hydrolysis, V_{max}, rather than K_m. This is consistent with the increased length of the template DNAs, allowing more rapid ATP hydrolysis before the translocase reaches the end of the DNA fragment. This form of length dependence is similar to that observed for bona fide helicases such as T4 gene 41 (68). In addition, Saha et al. performed an elegant experiment in which they showed that RSC and Sth1 have similar activities on short circular DNA molecules to longer linear ones. All of these approaches are consistent with these activities functioning as ATP-dependent DNA translocases. However, it should be noted that ATP hydrolysis need not necessarily provide a direct measure of DNA translocation.

In order to obtain additional evidence for DNA translocation, Saha et al. also used a triplex oligonucleotide displacement assay. They found that both Sth1p and RSC are capable of displacing TFOs in an ATP-dependent reaction. Although triplex displacement has been used previously to monitor the action of ATP-dependent DNA translocases and helicases (23, 39, 45), it is conceivable that TFOs could be displaced by other means. As a control for this, Saha et al. showed that triplex displacement is reduced from a triplex DNA that lacks double-stranded extensions and is still possible on one that contains a nick at the duplex-to-triplex interface.

Our studies of ISWI are entirely consistent with those described by Saha et al. and provide additional support for the model they present. We obtained two lines of evidence that support the involvement of a processive activity in triplex displacement. Firstly, triplex displacement decays with distance from the nucleosome. As an epitope within the H4 tail has been defined as playing a key role in the stimulation of ISWI ATPase activity, it is likely that at least a proportion of the ISWI protein functionally associates with this part of the histone octamer (13, 33). The fact that the TFO displacement decays with distance from this epitope supports a model in which translocation is orientated away from the nucleosome. Additional evidence for this orientation is provided by the observation that the insertion of 5- or 10-bp gaps in the 3′-5′ strand between the nucleosome and the triplex prevents triplex displacement.

In contrast to the effect of gaps in the 3′-5′ strand, the introduction of 5- or 10-bp gaps in the 5′-3′ strand has no effect on the ability of ISWI to function in triplex displacement assays. These observations are most readily explained if ISWI exhibits a strand-specific translocase activity. Strand specificity is a property of bona fide DNA helicases, which can be separated into two groups, 5′-3′ or 3′-5′, reflecting the polarity in which DNA transport occurs (10). Many members of the SF2 group, to which the ATP-dependent chromatin-remodeling activities are most closely related, display a 3′-5′-strand specificity. Our observation that 3′-5′, but not 5′-3′, gaps have an effect is consistent with this. Further evidence supporting the ability of remodeling activities to function on a single DNA strand stems from the observations that the ATPase activity of RSC (9), Sth1 (51), and ISWI (Fig. 3) is stimulated by single- as well as double-stranded DNA.

It is interesting to note that the ability of ISWI to function in

the displacement of triplexes located up to 40 bp from the edge of a nucleosome bears similarities to studies of the yeast Mot1 protein. This protein has homology to the ATP-dependent remodeling activities and functions to displace the TATA binding protein from DNA in an ATP-dependent reaction. Mot1-driven TATA binding protein displacement requires contact with a DNA "handle" adjacent to the TATA box, like ISWI, and the introduction of single-stranded DNA gaps does not prevent Mot1 functioning (16).

Although the observations of Saha et al. together with the evidence presented here suggest to us that ISWI, RSC, and Sth1 are likely to function as ATP-dependent DNA translocases, it is important to note that it has not yet been possible to directly monitor the motion of any remodeling activity along DNA or determine the translocation rate as has been possible with *Eco*R124 (23). Until this has been achieved, it remains a formal possibility that ATP-dependent remodeling activities distort DNA in some way that does not involve translocation. This will be technically difficult, as it appears that RSC and Sth1 have a processivity limited to 80 bp (51), while in the case of ISWI, our own assays suggest that ISWI processivity is limited to 40 bp. This is a relatively short distance compared to previously characterized ATP-dependent nucleic acid translocases such as gp14 (68) and *Eco*R124I (58). In addition, this processivity limit is typically reached as a result of a stochastic dissociation event occurring at distances distributed around a mean length. This is expected to give rise to an exponential decay in processivity that fits to an equation hyperbolic for cofactor length. However, the work presented here and much of the data obtained by Saha et al. suggest that the length dependence displayed by these chromatin-remodeling enzymes is more abrupt than would be expected for an exponential decay process. This, combined with the fact that highly processive motion over long distances may not be required for the local distortion of chromatin structure, raises the possibility that a more precise mechanism regulates the processivity of remodeling enzymes. Other helicases, such as the RecBCD enzyme, have been observed to translocate in a stepwise fashion (4). Our observations of DNA binding, double- and single-stranded ATPase activity, and triplex displacement are all consistent with a processivity limit for ISWI of approximately 40 bp. However, unlike RecBCD, our observations would suggest that ISWI is able to dissociate from the template after each cycle of translocation.

Interestingly, Sth1 and ISWI appear to have different processivity limits. Whereas RSC and Sth1 appear to be capable of translocating for 80 bp, for ISWI, both the ATPase assays and triplex displacement assays suggest that the processivity of ISWI is limited to approximately 40 bp. This could explain differences in the activities of the two enzymes. While RSC is able to disrupt nucleosomes in addition to moving them along DNA, ISWI appears to be more specialized toward nucleosome mobilization. The distortion of less DNA by ISWI may suit it for a role in nucleosome mobilization without nucleosome disruption. It is also interesting that the 40-bp limit for ISWI is compatible with the average nucleosome spacing in many higher eukaryotes.

How might a DNA translocase activity stimulate nucleosome mobilization? Our data were generated by using chromatin templates, which provide a means of orientating the distortion of DNA driven by an ATP-dependent remodeling activity, in this case, ISWI, relative to that of a nucleosome. As ISWI requires residues 16 to 19 of the H4 tail for maximal ATPase activity, it is likely that the ISWI retains contact with at least this part of the nucleosome during ATP hydrolysis. Translocation of the ISWI protein along DNA, while it remains associated with the histone octamer, could cause DNA to be injected onto the surface of nucleosomes. This would result in the generation of a loop or bulge of DNA on the surface of the nucleosome, which might then be able to transit around the surface of the octamer in a fashion similar to that proposed for the translocation of SP6 RNA polymerase around nucleosomes (56).

Since our results suggest that ISWI has strand specificity, translocation would be expected to follow one DNA strand around the helical DNA backbone. A key consequence of this would be the generation of superhelical torsion between the translocase and the site at which DNA contacts the nucleosome surface. Consistent with this suggestion, the generation of superhelical torsion on chromatin templates by ISWI was previously detected (35). This provides a second means by which DNA might be moved over the surface of the nucleosome. Local defects to the superhelical twist of DNA on the nucleosome might be transmitted incrementally over the surface of the nucleosome, as proposed previously (62). This is also supported by the distortion of DNA in high-resolution structures of the nucleosome (17). Where rotation of the DNA on the surface of the nucleosome is not favored, for example, in the presence of strong rotational phasing, a buildup of torsion might be capable of impeding nucleosome mobilization. Consistent with this, it has been observed that the ySWI/SNF complex is inefficient in remodeling nucleosomes on small minicircles that cannot readily accommodate changes in twist (28). It has also recently been observed that the presence of nicks in a DNA template located at sites that could relieve torsion as it accumulates as a consequence of ISWI translocation stimulates the ability of ISWI to function in nucleosome mobilization (40). This is also consistent with a role for torsion in remodeling.

Neither of these mechanisms are necessarily mutually exclusive, and it is even possible to imagine a combination of the two. It is possible that the structural characteristics of the DNA, especially its flexibility and inherent curvature, play an important role in determining what combination of the two mechanisms is involved. Whatever mechanism is involved, it remains unclear why ISWI appears unable to move nucleosomes from the ends of some DNA fragments such as the one used in this study. It appears to be a general property of the movement of nucleosomes on short DNA fragments that they tend to accumulate toward DNA ends (25, 47). One explanation for this has been that there is an energetic penalty associated with the crossover of DNA duplexes entering and/or exiting the nucleosome in close proximity that is absent for nucleosomes at the ends of DNA fragments (52). However, this phenomenon has been characterized on only very few DNA fragments to date and we have observed cases where nucleosomes positioned at the ends of some DNA fragments can be relocated to other positions by ISWI or thermal incubation (A. Flaus, unpublished data). Thus, it is possible that the stability of end-positioned nucleosomes is a result of the

combined energetics of DNA-sequence-dependent nucleosome positioning and the absence DNA duplex crossover at the entry and/or exit to the nucleosome such that some nucleosomes, like the one used here, strongly resist movement.

Although the ISWI protein provides an excellent model system to study mechanisms for ATP-dependent chromatin remodeling due to the fact it can be expressed as a recombinant protein and has ATPase activity that is targeted to a specific site on the histone octamer, it is likely that the means by which other ATP-dependent remodeling activities function may differ, at least to some extent. Indeed, the ISWI protein itself is normally found to be associated with other proteins that alter the way in which it moves mononucleosomes (19). Thus, it will also be important to determine how the assembly of ISWI into native complexes alters its action in nucleosome mobilization.

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