Nucleic Acids Research, 1997, Vol. 25, No. 18 **3733–3742**

Binding of NF1 to the MMTV promoter in nucleosomes: influence of rotational phasing, translational positioning and histone H1

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Received January 22, 1997; Revised and Accepted July 18, 1997

ABSTRACT

To analyse the role of rotational orientation and translational positioning of nucleosomal DNA on transcription factor binding we have generated a series of mutant MMTV promoters containing insertions of various lengths between the hormone-responsive region and the binding site for NF1. These various MMTV promoter fragments were assembled in mononucleosomes and used for structural studies and binding experiments. We show that the insertions change the rotational phase and translational positioning of the NF1 site as predicted if the sequences upstream of the insertion site were the main determinants of nucleosome phasing. In band shift experiments with recombinant NF1 we cannot detect binding of the protein to NF1 sites included within the limits of a nucleosome, independent of their rotational orientation. Moving the NF1 site closer to the nucleosome border also did not permit NF1 binding. This behaviour probably reflects the way NF1 binds DNA, namely it almost completely surrounds the circumference of the double helix establishing a large number of contacts with the bases and the backbone. In contrast to the wild-type and short insertion mutants, NF1 bound readily to nucleosomes containing 30 or 50 bp insertions which placed the NF1 site at the nucleosome edge or within linker DNA. NF1 binding to the linker DNA was unaffected by incorporation of histone H1 into the nucleosome particle. These findings are discussed in relation to chromatin remodelling initiated by steroid hormones during induction of the MMTV promoter.

INTRODUCTION

One open question in the context of eukaryotic gene regulation is how transcription factors gain access to their cognate sites on organized chromatin nuclear DNA. Access is restricted by the various levels of DNA packaging in chromatin. The higher order structure of chromatin is poorly defined in molecular terms, but wrapping of the double helix around the histone octamer in nucleosomes results in hindrance of DNA binding proteins. Even if chromatin is a dynamic structure (1), the tight contacts between DNA and the core histones represent a barrier for recognition of specific sequences by regulatory proteins. This obstruction is particularly strong when the DNA double helix is precisely positioned on the surface of the histone octamer, a situation often encountered in promoter and enhancer regions of regulated genes (2).

Two kinds of genetic evidence support a repressive role of chromatin on gene activation: the phenotype of mutations in the histone genes and the identification of yeast genes able to counteract the repressive effect of chromatin. This latter class of genes includes the SWI–SNF complex of *Saccharomyces cerevisiae* and their homologues in other species, which form large molecular complexes with DNA-dependent ATPase activity (3). Mutations in these genes lead to pleiotropic effects affecting many inducible genes and suppressors of this phenotype often map to chromatin proteins. On the other hand, artificially generated nucleosome depletion, as well as certain mutations in the N-terminal domains of the core histones, lead to increased constitutive activity of several inducible genes in the absence of inducers or upstream activating sequences (UAS) (4). These findings suggest that one of the functions of UAS and the *trans*-acting factors they bind is to relieve repression of the promoter due to their organization in nucleosomes.

Two questions arise. First, what is the nature of the obstruction imposed by nucleosomes on DNA binding proteins? Second, how do certain regulatory proteins nevertheless manage to recognize their cognate sequences organized in chromatin? To address these questions we have focused on the mouse mammary tumor virus (MMTV) promoter, which is silent in the absence of steroid hormones but highly active after induction with glucocorticoids or progestins (5 and references therein). Induction is mediated by a 150 bp hormone regulatory region encompassing several hormone-responsive elements (HREs), a binding site for members of the NF1 family of transcription factors and two octamer motifs. All these *cis*-acting elements are required for full hormonal induction of the promoter and there is a strong functional synergism between the hormone receptors and NF1 (6–8). This synergism cannot be reproduced under cell-free conditions using free MMTV DNA as template for *in vitro* transcription (8,9). Indeed, on free promoter DNA the hormone receptors and NF1 compete for binding to their adjacent sites (8).

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The MMTV promoter is organized into an array of positioned nucleosomes (10) and several groups have produced evidence supporting a role for chromatin in regulation of MMTV transcription by steroid hormones (for a review see 11). Although the existence of multiple translational frames has been reported based on analysis of mononucleosomal DNA fragments (12), genomic footprinting experiments detect one major population of positioned nucleosomes which covers the region of the HREs and the NF1 site and may preclude binding of NF1 (10,13). In the absence of hormones there is no evidence for any sequence-specific factor bound to the promoter in genomic footprinting experiments (13). In particular, the NF1 site is not occupied (13,14). Following hormone treatment all the HREs as well as the NF1 site are occupied *in vivo* and the positioned nucleosome over the promoter remains in place (13), although the underlying DNA sequences become more sensitive to cleavage by nucleases or nucleolytic agents (10,13,15). Therefore, one explanation for the differences between *in vivo* and *in vitro* results in terms of the synergism between receptors and NF1 is that binding of the hormone–receptor complex to the promoter changes the orientation of the nucleosomal DNA, exposing the previously inaccessible NF1 site.

Attempts to reproduce the *in vivo* MMTV behaviour with reconstituted nucleosomes have been only partly successful. Positioned nucleosomes can be generated with short pieces of the MMTV promoter and these nucleosomes bind hormone receptors (16), but they do not bind NF1, even in the presence of bound receptors (17). The ability of the hormone receptors to recognize HREs depends on the rotational orientation of the major groove of DNA, as only those HREs are bound whose major groove points outwards (17,18). The orientation of the major groove of the NF1 site in the MMTV nucleosome is inappropriate for binding, but no information is available as to the relative contribution of rotational versus translational positioning on NF1 binding. Recent results with an artificial sequence assembled into nucleosomes suggest that both parameters are important in determining the apparent affinity of NF1 for its target sequences (19).

To study this question in the context of the native MMTV promoter sequence we have generated a series of insertions between the hormone-responsive region and the NF1 site which should change the rotational setting as well as translational positioning of the NF1 binding site relative to the nucleosomal dyad axis. Here we present the structural characterization of these sequences assembled into mononucleosomes and describe their NF1 binding properties. By mapping the contacts of NF1 with the bases and with the sugar–phosphate backbone on the MMTV promoter we provide a plausible explanation for the inability of NF1 to bind to a nucleosomally organized promoter, no matter the rotational setting. When the NF1 binding site is displaced to the linker DNA we observed binding of NF1, which is not inhibited by incorporation of histone H1 into the complex.

MATERIALS AND METHODS

Chromatin preparation

Long chromatin fragments depleted of histone H1 and non-histone proteins were prepared from rat liver as described (20). Briefly, rat livers were homogenized in TES buffer [0.5 M sucrose, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 2 mM EDTA, 1 mM dithiothreitol (DDT) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in 15 mM Tris–HCl, pH 7.4] plus 0.5% Triton X-100. Three volumes of 2.3 M sucrose in the same buffer without detergent were added to the homogenate and the mixture was layered onto a TES buffer plus 2.3 M sucrose cushion in SW28 rotor ultracentrifuge tubes (Beckman Inc.). Nuclei were pelleted at 27 000 r.p.m. for 2 h at 4°C and resuspended in TES buffer plus 0.35 M sucrose. Nuclei (10 mg) in a total volume of 2 ml were digested with 10 U micrococcal nuclease (Sigma Inc.) in the presence of 5 mM $CaCl_2$ for 10 min at 37°C. The reaction was stopped by addition of 200 µl 0.5 M EDTA and the nuclei pelleted at 4000 *g* for 2 min at 4°C. The pellet was resuspended in 1 ml 0.2 mM EDTA, pH 7.4, and the nuclei disrupted by repeated pipetting. Undigested chromatin and nuclear matrix were removed by centrifugation at 4000 *g* for 5 min at 4°C. Between 40 and 60% of the absorption at 260 nm from the starting material was recovered in the supernatant, containing soluble chromatin fragments. Histone H1, non-histone proteins and the mononucleosomal and dinucleosomal fractions were removed by gel filtration chromatography using an 80 cm column of Bio-Gel A0.5m equilibrated with 450 mM NaCl, 0.5 mM EDTA and 0.2 mM 2-mercaptoethanol in 5 mM Tris–HCl, pH 7.4. The column was run at a flow rate of 25–30 ml/h. The exclusion fraction, containing chromatin fragments of 3–100 nucleosomes in length, was collected and kept at 4°C. The protein composition and integrity of the fractions were checked by SDS–PAGE.

Nucleosome reconstitution and purification of reconstituted material

The ^{32}P-5′-end-labelled DNA fragment used for most reconstitution experiments contains the MMTV promoter region from –203 to –19. For the band retardation assay including histone H1 a longer fragment from –234 to –19 was used. About 100 ng of end-labelled DNA fragment was mixed with 30 µg chromatin in the presence of 1.2 M NaCl in TED buffer (10 mM Tris–HCl, 1 mM EDTA, 1 mM DDT, pH 7.4) in a total volume of 120 µl and dialysed against 2 M NaCl in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) including 1 mM 2-mercaptoethanol for 2 h at 4°C. The salt concentration was then reduced to 100 mM NaCl by gradient dialysis overnight at 4°C. Purification of reconstituted material was by glycerol gradient ultracentrifugation using a linear gradient from 5 to 30% glycerol in 50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 mM DDT and 0.1 mg/ml bovine serum albumin (BSA). Centrifugation was performed in a SW60 rotor (Beckman Inc.) for 7 h at 55 000 r.p.m. and 4°C. Fractions of 200 µl were collected from the top of the gradient and measured for radioactivity.

Exonuclease III digestion

The reconstituted octamer was digested with 200 U exonuclease III at 25°C in TGA buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 mM DDT, 10% glycerol) supplemented with 90 mM NaCl and 5 mM $MgCl_2$ in a total volume of 200 µl. After the indicated incubation times aliquots of 65 µl were taken out of the reaction mixture and added to 35 µl DNase I stop buffer (62.5 mM EDTA, 2.5% SDS). The samples were incubated with 50 U proteinase K at 37°C for at least 2 h. Proteins were removed by phenol/chloroform/isoamyl alcohol extraction and the remaining DNA was precipitated with isopropanol. After washing with 80% ethanol and drying the radioactivity was determined and equal amounts were analysed on a 6.5% acrylamide–7 M urea gel.

Hydroxyl radical cleavage

Hydroxyl radical cleavage reactions of the reconstituted material were done by mixing the samples in 170 µl reaction buffer (10 mM Tris–HCl, pH 7.4, 100 µg/ml BSA, 5% glycerol) with 10 µl each of solution A [18.5 mM $(NH_4)_2Fe(SO_4)_2$, 37 nM EDTA], solution B (180 mM sodium ascorbate) and solution C (2.22% H_2O_2). After 5 min incubation at room temperature the reaction was stopped by adding 45 µl 270 mM thiourea. Removal of proteins, precipitation of DNA and electrophoretic analysis were performed as described for the exonuclease III digestion experiments.

Band retardation assays

Free DNA and/or reconstituted material were incubated with different amounts of NF1 in TGA buffer (see exonuclease III digestion experiments) containing 90 mM NaCl, 1 µg poly(dI·dC) and 3 µg/µl BSA. Binding reactions were performed for 20 min at room temperature in a total volume of 20 µl. Bound and unbound material was separated in a 0.5× TBE (50 mM Tris base, 45 mM boric acid, 0.5 mM EDTA)–5% polyacrylamide gel run at room temperature. For band retardation assays including histone H1 the linker histone was added before 1 µl NF1 (0.4 µg) and preincubated for 10 min at room temperature. The molar ratio of H1 to nucleosomes was 1:1.

Interference experiments

To identify contacts between NF1 and DNA, interference experiments with DNA modification by either ethylnitrosourea (for phosphate contacts), $KMnO_4$ (for T contacts) or DMS (for G contacts) were performed. As DNA, a MMTV fragment including the region from –83 to –32 (GNO-WT), which contains the NF1 binding site, was used. Either the upper or lower strand was radioactively labelled at the 5′-end.

Ethylnitrosourea modification. Aliquots of 100 ng labelled single strand (GNO-WT oligo) in 100 µl 50 mM sodium cacodylate, pH 7.0, was mixed with 100 µl saturated ethylnitrosourea solution in ethanol and incubated at 50°C for 90 min. For hybridization the single strand was precipitated by ethanol and resuspended in 20 µl TE containing 300 mM NaCl and 300 ng counter strand.

$KMnO_4$ modification. Aliquots of 100 ng labelled single strand (GNO-WT oligo) in 5 µl 30 mM Tris–HCl, pH 8.0, were incubated with 20 µl freshly prepared 1 mM $KMnO_4$ solution for 5 min at room temperature. The hybridization was performed as described for ethylnitrosourea modification.

DMS modification. To 200 ng labelled double-stranded GNO-WT oligo in 200 µl 50 mM sodium cacodylate, pH 7.0, was added 1 µl dimethyl sulphate (DMS), followed by incubation for 2 min at room temperature.

All modifications were stopped by ethanol precipitation.

Band retardation assay. After modification a band retardation assay (as described before) containing 2 µl (~800 ng) NF1 was performed. DNA and DNA–NF1 complexes were electroblotted on a DE-81 membrane (Whatman). The bands were cut out and eluted with TE containing 1.5 M NaCl for 2 h at 70°C.

Cleavage and analysis of modified DNA. Ethylnitrosourea-modified DNA was resuspended in 15 µl phosphate buffer, pH 7.0, and incubated with 2.5 µl 1 N NaOH at 90°C for 30 min. $KMnO_4$ and DMS-modified DNA was resuspended in 90 µl H_2O and incubated with 10 µl piperidine for 30 min at 90°C. The remaining DNA was precipitated with ethanol. After washing with 80% ethanol and drying the radioactivity was determined and equal amounts of radioactivity were analysed on a 6.5% acrylamide–7 M urea gel.

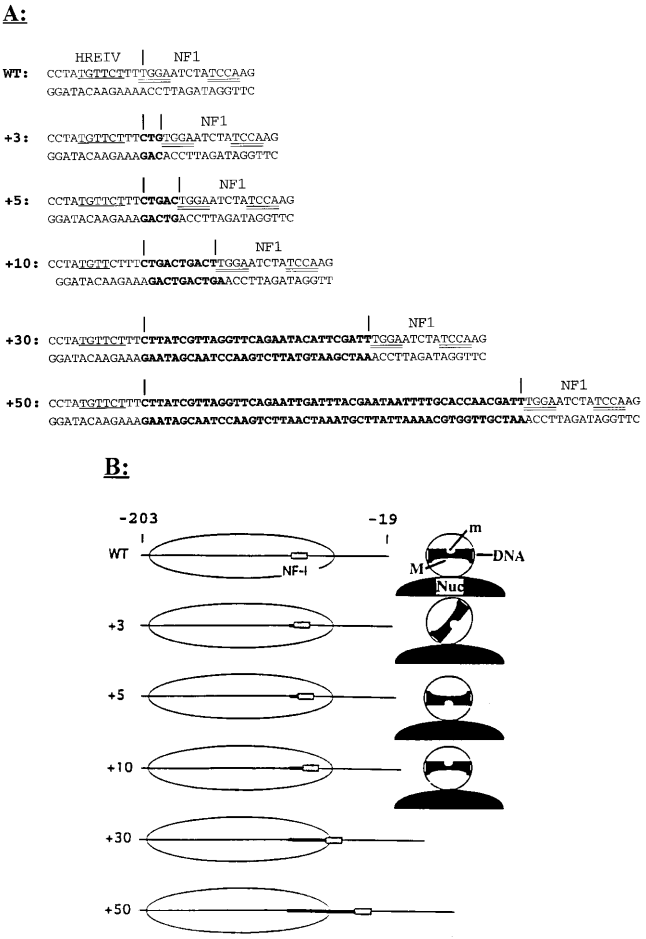

Figure 1. Schematic representation of the insertion mutants and their predicted nucleosomal structure. (**A**) Sequence of the insertion mutants. HRE4 (single underline) and NF1 binding site (double underline) are shown and the site of the insertion at –76 is marked by a vertical line in the wild-type sequence. The numbers on the left side indicate the number of inserted base pairs and the sequence of the insertions is printed in bold. (**B**) Predicted translational position and rotational orientation of the NF1 site. The left half shows the location of the NF1 binding site relative to the nucleosome borders. The right half shows the orientation of the NF1 binding site relative to the nucleosome surface. Nuc, nucleosome; M, major groove; m, minor groove at the conserved palindrome.

RESULTS

Structural characterization of mononucleosomes with various insertions

The insertion mutants used for this study are shown in Figure 1A. The fragment used for nucleosome reconstitution and the major translational frame observed with wild-type MMTV promoter sequences (17) are shown in Figure 1B (top). The NF1 site, indicated by a box, is located 20 bp upstream of the proximal nucleosome border and additional bases were inserted just upstream of the NF1 site (thick line). The scheme on the right represents a section of the double helix at the NF1 site showing

that the major groove over the conserved palindrome (double underlined in Fig. 1A) is pointing to the histone octamer. Assuming that the sequences upstream of the insertion site are sufficient to establish nucleosome positioning, the predicted effect of the insertions on rotational orientation and translational position of the NF1 binding site is indicated (Fig. 1B). The 3 bp insertion should change the orientation of the major groove by ~105°, whereas the 5 bp insertion should generate an orientation opposite to that found in the wild-type sequence. The longer insertions should maintain the rotational setting and move the NF1 binding site away from the nucleosomal dyad axis and into the linker DNA. To confirm these predictions we analysed reconstituted nucleosomes by digestion with exonuclease III and by hydroxyl radical cleavage.

Exonuclease III digestion was performed to determine the rotational orientation of the DNA double helix on the surface of the histone octamers. The cleavage pattern shows that the promoter sequences with various insertions were all assembled in nucleosomes with similar rotational phase as the wild-type promoter. The same pattern of bands spaced by ~10 bp was found upstream of the insertion site (Fig. 2, cleavage between –80 and –100). The rotational orientation of the NF1 binding site is changed by the insertions in the predicted way. In the wild-type configuration there are two clusters of cleavage signals on both ends of the NF1 site corresponding to the two halves of the palindrome (Fig. 2A). In the 10 bp insertion mutant a similar distribution of cleavage sites over the the NF1 site is found. In the 3 bp insertion mutant the proximal cluster of cleavage sites moves towards the centre of the NF1 site and the distal cluster is now located upstream of the NF1 binding site. This trend is more pronounced in the 5 bp insertion mutant. In the longer insertion mutants there is no clear 10 bp spacing between clusters of clevage sites over the NF1 binding site (Fig. 2B). In the 30 bp insertion mutant the NF1 site is located immediately adjacent to the last cluster of the wild-type 10 bp cleavage pattern and new clusters of cleavage sites appear over the relevant region. This is particularly obvious with the long digestion time because the 30 bp insertion was less digested than the wild-type and the 50 bp insertion. Upstream of the insertion site at –76 the 10 bp cleavage pattern observed is shifted by 1–2 nt, as if the rotational setting in this region has been slightly altered. In the 50 bp insertion mutant the NF1 site is in a region without clear clevage periodicity, probably reflecting a location outside the nucleosome core particle. The 10 bp cleavage pattern upstream of the insertion site is as in the wild-type nucleosome.

A more precise determination of the orientation of the NF1 binding site in nucleosomes containing promoter mutants with short insertions was accomplished in hydroxyl radical cleavage experiments (Fig. 3A). This agent cleaves the sugar–phosphate backbone of the DNA at the minor groove and prefers those sites with an exposed and widened minor groove pointing outwards. As reported previously (17), in the wild-type configuration the two halves of the palindromic NF1 site are oriented with the minor grooves pointing outwards and, therefore, coincide with maxima of cleavage efficiency (Fig. 3A, lower tracing). A minimum of cleavage efficiency coincides with the centre of the NF1 site. In the 3 bp insertion mutant this minimum is shifted upstream and in the 5 bp insertion mutant it lies over the distal half of the palindrome, while a new minimum coincides with the proximal half. Thus in this configuration both halves of the palindrome are oriented with their major grooves pointing outwards.

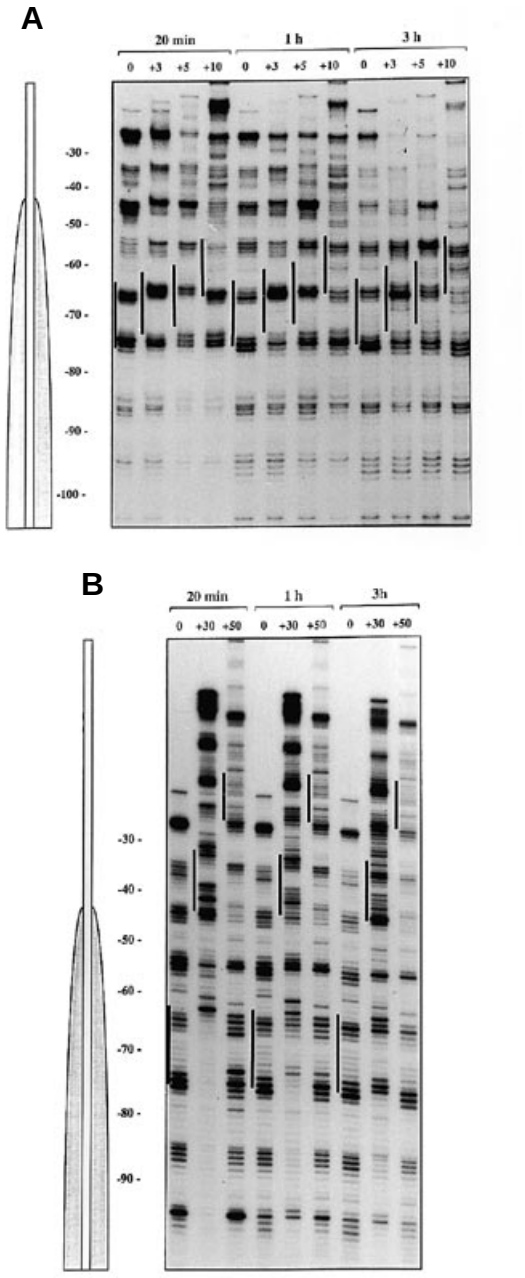

Figure 2. Exonuclease III digestion. Reconstituted mononucleosomes were digested with 200 U exonuclease III at 25°C for the time intervals indicated at the top. Digestion products were analysed on a denaturing polyacrylamide–urea gel. The number of base pairs inserted in the MMTV fragment is indicated on top of each lane. The position of the NF1 binding site is indicated by black bars on the autoradiograms to the left of each lane. On the left side of the figure a sketch of the wild-type MMTV fragment with the dominant position of nucleosome B is shown. (**A**) MMTV wild-type and short insertions (WT, +3, +5 and +10 bp). (**B**) MMTV wild-type and long insertions (WT, +30 and +50 bp)

In the 10 bp insertion mutant the wild-type orientation of the NF1 site is restored with a cleavage minimum over the centre of the palindrome (Fig. 3A, top tracing). The pattern of cleavage upstream of the insertion site and over the nucleosomal dyad axis is identical in all cases (data not shown), suggesting that translational phasing of the nucleosome is not altetered by the short insertions.

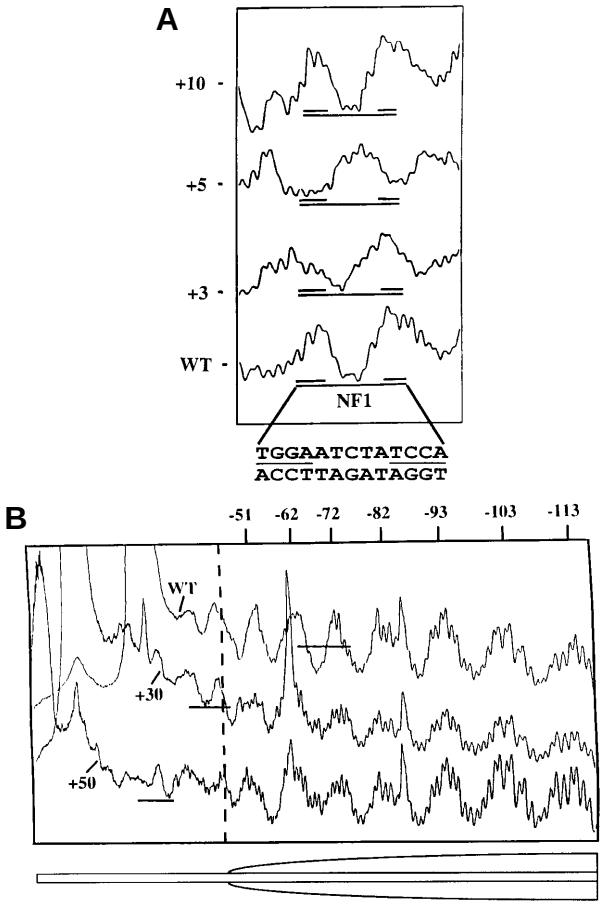

Figure 3. Hydroxyl radical cleavage. Reconstituted mononucleosomes were cleaved using hydroxyl radicals and the products analysed on a denaturing polyacrylamide–urea gel. The figure shows intensity scans of the autoradiogram containing the region of the NF1 binding site, which is indicated by black bars. (**A**) MMTV wild-type and short insertions (WT, +3, +5 and +10 bp). Only the region corresponding to the NF1 binding site is shown. The scans are centred over the NF1 palindrome to allow a better comparison. The sequence of the NF1 binding site is shown at the bottom. The number of base pairs inserted in the MMTV fragment is indicated to the left. (**B**) MMTV wild-type and long insertions (WT, +30 and +50 bp). The scheme at the bottom indicates the dominant position of nucleosome B (shadowed). The proximal border of the nucleosome is indicated by a vertical broken line.

The hydroxyl radical cleavage pattern of the longer insertions shows that the general structure of the nucleosomal particle upstream of the insertion site is well preserved (Fig. 3B). The virtual identity of cleavage site intensity suggests that the dyad axis of the nucleosome has been maintained in the majority of the nucleosome population despite the long insertions. Downstream of the insertion site the 10 bp periodicity of the pattern is only maintained until the border of the wild-type nucleosome, although there are changes in the intensity of individual sites due to changes in nucleotide sequence. These results confirm the prediction that the 30 bp insertion leads to displacement of the NF1 site to the linker DNA at the edge of the nucleosome, whereas the 50 bp insertion places the NF1 site well into the linker DNA.

Binding of NF1 to mononucleosomes with short insertions

The binding of NF1 to mononucleosomes was analysed in band shift assays with recombinant histidine-tagged NF1 produced in baculovirus and purified by chromatography on Ni–NTA agarose columns. The recombinant NF1 bound as a homodimer to short oligonucleotides containing the NF1 consensus sequence (data not shown). It also bound very efficiently to a labelled wild-type MMTV promoter fragment, generating a complex which migrates slower than the mononucleosome (Fig. 4A). With the very high concentrations of recombinant NF1 used in this experiment a second even slower migrating complex is found (DNA+2NF1 in Fig. 4A). Since we do not see additional NF1 footprints (see Fig. 6) and similar slower complexes were seen with short NF1 oligonucleotides (data not shown), we interpret this complex as resulting from binding of an additional NF1 dimer through protein–protein interactions. The wild-type MMTV nucleosome included in the binding reactions did not bind NF1, as demonstrated by the lack of a corresponding ternary complex (see below). Moreover, even when >90% of the free DNA was shifted into the slow migrating NF1-retarded complexes, the intensity of the free nucleosomal band was unaffected (Fig. 4A, compare lanes 3 and 5). Under these conditions a small proportion of the labelled DNA is trapped in aggregates and does not enter the gel. These findings confirm our previous report that NF1 cannot bind to its cognate site in the MMTV promoter when the promoter is organized in nucleosome core particles (17).

A similar behaviour was found for nucleosomes reconstituted with MMTV promoters carrying short insertions. None of the insertions had an influence on the affinity of NF1 for the free DNA fragment. In addition, with none of the corresponding mononucleosomes did we observe binding of NF1: no ternary complex was formed and the band of free nucleosome was unaffected by addition of large amounts of recombinant NF1. We conclude that NF1 cannot bind to its cognate site on the MMTV promoter in mononucleosomes, even when the orientation of the major groove is opposite to that found in the wild-type configuration. An explanation for this finding may be provided by mapping of contacts between NF1 and its cognate DNA binding site in the MMTV promoter (see below).

Binding of NF1 to mononucleosomes with long insertions

We next analysed the NF1 binding properties of mononucleosomes reconstituted with MMTV promoters carrying long insertions, which should place the NF1 site outside the nucleosome core (Fig. 4B). There was no influence of the insertions on the affinity of NF1 for free MMTV promoter fragments. However, nucleosomes reconstituted with promoters carrying the 30 bp insertion gave rise to a ternary complex with NF1 migrating slower than the complex with free DNA. Moreover, under conditions where a significant amount of the nucleosomal DNA was included in this ternary complex there was a corresponding reduction in the free nucleosomal band and no free DNA band appeared (Fig. 4B, compare lanes 7 and 8). This suggests that binding of NF1 to the nucleosome carrying the insertion does not destabilize the nucleosomal core particle. The slow migrating weak band observed in the +30 nucleosome (Fig. 4B, lane 8) was not further characterized, but it may correspond to a complex of free DNA with two NF1 homodimers.

A similar situation was found with the promoter carrying the 50 bp insertion. However, the pattern with this construction was more complex, as the original nucleosome population was a mixture containing two dominant electrophoretic bands and two weak slower migrating bands (Fig. 4B, lane 11). It seems that both

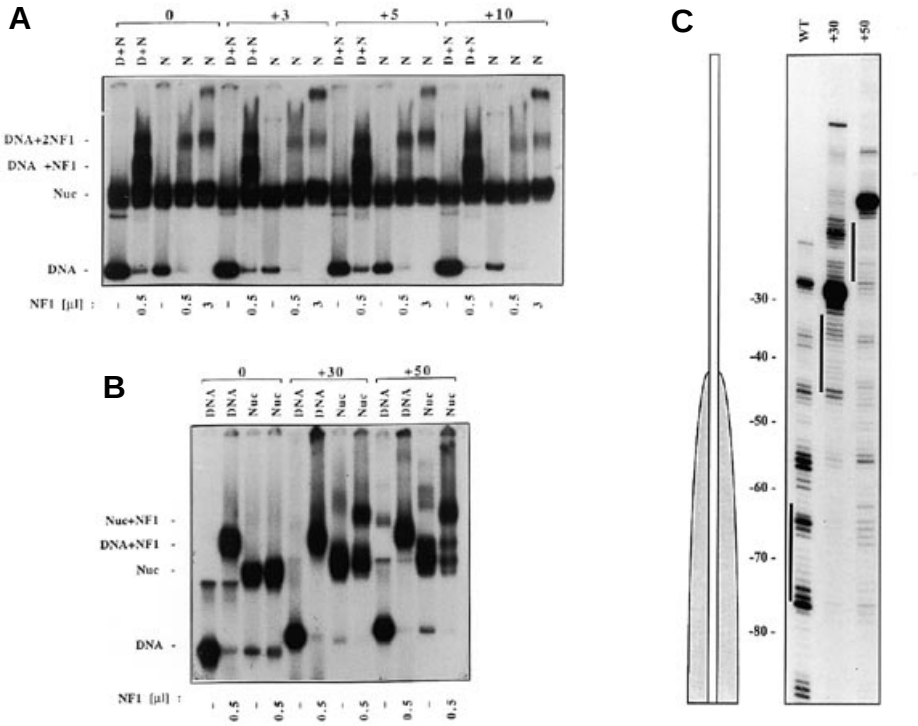

Figure 4. Binding of NF1. Autoradiograms of the band retardation assays. Binding reactions with NF1 were allowed to proceed for 15 min and the samples analysed on a 5% polyacrylamide–10% glycerol–0.5× TBE gel. (**A**) Influence of rotational positioning on NF1 binding. Reconstituted mononucleosomes containing either the wild-type MMTV fragment or fragments with one of the short inserts (+3, +5 or +10 bp, as indicated at the top of the figure) were mixed with the corresponding free DNA and incubated at room temperature with or without recombinant NF1 (amount of NF1 as indicated at the bottom of the figure; the concentration of the NF1 preparation was ~400 µg/ml). The identity of the bands is indicated on the left. DNA, free DNA; Nuc, nucleosome; DNA+NF1, complex of NF1 and DNA; DNA+2 NF1, complex of two NF1 molecules and DNA. (**B**) Influence of translational positioning on NF1 binding. Reconstituted mononucleosomes (Nuc), containing either wild-type MMTV fragment or fragments with one of the long inserts (+30 or +50, as indicated at the top of the figure), as well as the corresponding free DNA fragment (DNA), were incubated at room temperature with or without 0.5 µl (~200 ng) recombinant NF1. The identity of the bands is as indicated in (A) and Nuc+NF1 is a ternary complex of nucleosome and NF1. (**C**) Exonuclease III footprint. Nucleosomes assembled on the wild-type sequence and on the +30 and +50 bp insertion mutants were incubated with NF1 and digested with exonuclease III. In the wild-type sample only the stops generated by the positioned nucleosome are visible. In the 30 and 50 bp insertion samples an additional strong signal is detected just preceding the NF1 site (indicated by a thick bar to the left of the corresponding lane), which is not obvious in the absence of NF1 (see Fig. 2B).

main populations of nucleosomes were able to form a ternary complex with NF1, without generating free DNA bands.

The specificity of NF1 binding was additionally demonstrated in exonuclease III footprinting experiments (Fig. 4C). As previously reported, no NF1 binding could be detected with this sensitive technique using the wild-type promoter reconstituted into mononucleosomes. Binding of the protein should produce a signal just proximal of the NF1 site (marked by a vertical line left of the corresponding lane in Fig. 4C). Such a signal was missing in the wild-type sample, but clearly visible in the nucleosomes with the 30 and 50 bp insertions. Moreover, exonuclease III stops distal of the NF1 site were markedly reduced in nucleosomes with insertions, demonstrating that a significant proportion of these nucleosomes carry a bound NF1 protein. We conclude that nucleosomes assembled on the 30 and 50 bp insertions contain partially accessible NF1 sites.

Influence of histone H1 on binding of NF1 to mononucleosomes

Since NF1 appears to be able to interact with MMTV nucleosomes when its cognate site is positioned at the nucleosome edge or in the linker DNA and this region is assumed to be contacted by the linker histones (21), we next studied the influence of histone H1 on binding of NF1 to promoters with long insertions. Addition of stoichiometric amounts of histone H1 to mononucleosomes assembled on promoter fragments with long insertions generated a slower ternary complex containing the linker histone (Fig. 5). Interestingly, the heterogeneous populations of nucleosome core particles found with the 30 and 50 bp insertions were converted to a more homogeneously migrating population upon binding of histone H1 (Fig. 5, compare lanes 1 and 3 and 5 and 7). When NF1 was added a quaternary complex was formed migrating slower than the ternary complex of NF1 with the core particle (Fig. 5, compare lanes 2 and 4 and 6 and 8). The intensity of this quarternary complex containing H1 was equivalent to that of the ternary complex with the nucleosome core particle. These results suggest that the presence of histone H1 in the nucleosome does not interfere with NF1 binding to its cognate site on the edge of the nucleosome or in the linker DNA.

Mapping the contacts of NF1 with bases and phosphates

The finding that NF1 cannot gain access to the NF1 site within nucleosomes, even when the rotational orientation is changed to expose the major grooves of the two halves of the binding palindrome, prompted us to study the contacts between the protein and the double helix in more detail.

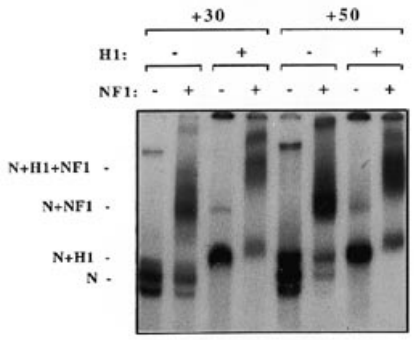

Figure 5. Influence of histone H1 on NF1 binding to nucleosomes containing long insertions. Nucleosomes assembled on the +30 and +50 bp insertion mutants incubated with or without histone H1 (+ or –, as indicated at the top of the figure) were incubated with or without NF1 (~400 ng, + or –, as indicated). The identity of the bands is marked to the left of the gel. N, nucleosome without H1; N+H1, nucleosome including H1; N+NF1, complex of nucleosome and NF1; N+H1+NF1, complex of nucleosome, H1 and NF1.

We first mapped the contacts of the protein with the N7 position of guanines in the major groove using DMS interference assays (Fig. 6A). Modification of any of the guanines in each half of the palindromic NF1 site interfered with binding and in both strands the effect of modifying the outer G was more pronounced than that of modifying the more central G. Similar findings have been reported for binding of NF1 to the adenovirus origin of replication (22). The central G of the palindrome in the lower strand is not contacted and we do not detect contacts to the N3 positions of adenines in the minor groove (Fig. 6A and data not shown). Thus the NF1 homodimer contacts four G·C base pairs.

We next determined the contacts of NF1 with the 5′-methyl group of thymines in the major groove, using the $KMnO_4$ interference technique (23). We found strong contacts to the two thymines in each half of the TGGA palindrome (one in each strand) and additional contacts with the four T residues flanking the upstream half of the palindrome (three in the upper strand and one in the lower strand) as well as with the T flanking the downstream half of the palindrome in the lower strand (Fig. 6B). These results show that the NF1 homodimer contacts five A·T base pairs within the palindrome and four flanking A·T base pairs.

Finally, we analysed the contacts of NF1 with phosphates using the ethylnitrosourea interference technique (24). Two clusters of four contacted phosphates were found in the outer part of each half palindrome with the sequence TTGG (Fig. 6C). In addition, a weak contact was found at the inner phosphate of each half palindrome in the opposite strand, adding to a total of 10 phosphate contacts.

A representation of the 23 contacts between NF1 and MMTV DNA is shown in Figure 7. As can be seen in the axial projection, the homodimer of NF1 surrounds the double helix almost completely (Fig. 7, bottom, right panel). This type of DNA sequence recognition is in marked contrast to that previously found for contacts between hormone receptors and HREs. For comparison we show the experimentally found contacts between the glucocorticoid and progesterone receptors and HRE4 of MMTV,

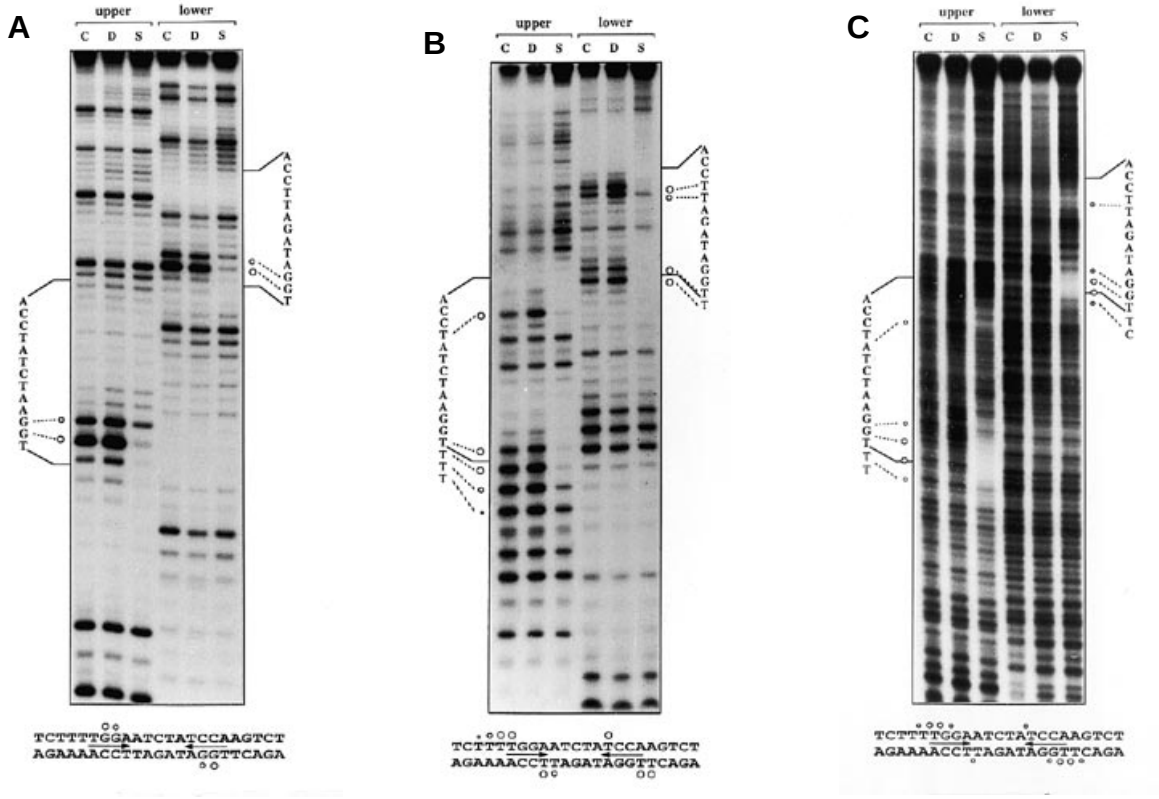

Figure 6. Contacts between NF1 and the DNA double helix. (**A**) DMS interference assay. (**B**) $KMnO_4$ interference assay. (**C**) Ethylnitrosourea interference assay. The relevant regions of the sequence of each strand are shown near the corresponding lanes. The contacted residues in the upper and lower strands are indicated by circles (large circles indicate strong protection, smaller circles indicate weaker protection). C, control lane without protein; D, free DNA; S, NF1-bound DNA. The sequence of the NF1 binding site with the mapped contacts is shown at the bottom.

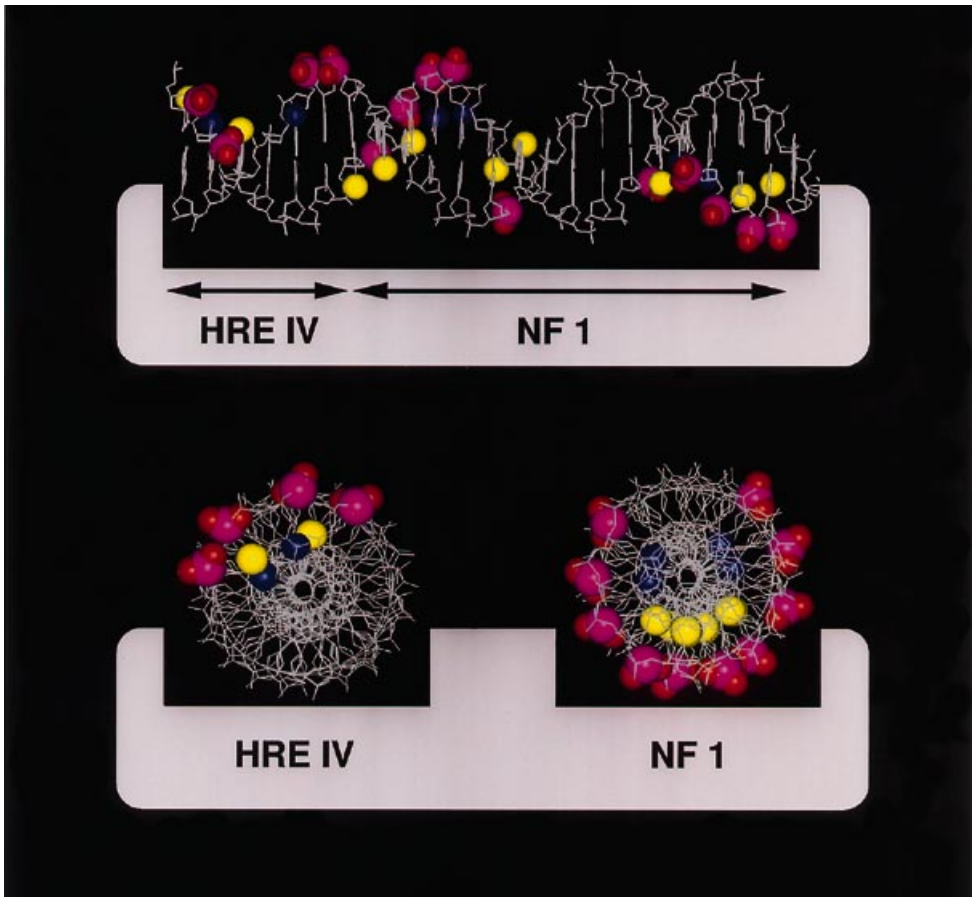

Figure 7. Schematic representation of the DNA contacts of the hormone receptor and NF1 with the MMTV sequences between –83 and –56. (Top) Lateral view of the sequence. The regions corresponding to HRE4 and the NF1 site are indicated. (Bottom) Axial view of the sequence. (Left) HRE4. (Right) NF1 site. Red/pink, phosphate contacts; blue, contacts with N7 position of guanines; yellow, contacts with methyl groups of thymines; grey, localization of the nucleosome.

which is located immediately upstream of the NF1 site (7,23). Only six contacts are found in this half palindrome and all are clustered within a narrow sector of the circumference of the double helix (Fig. 7, bottom left). This difference in DNA binding properties may explain the different affinities of hormone receptors and NF1 for nucleosomes (see Discussion).

DISCUSSION

Sequences upstream of the NF1 site determine translational and rotational positioning of nucleosome B

We have previously shown that the DNA sequence of the MMTV promoter contains the information required to drive positioning of an octamer of histones to a preferential location, between nucleotides –190 and –45 (25). Insertions at position –146 did not disturb the proximal border of this nucleosome, suggesting that sequences downstream of –146 are essential for determining the 3′-border of the histone octamer (26). One of the conclusions which can be drawn from the experiments reported above is that the main determinants for the translational position of nucleosome B are located upstream of the NF1 binding site. This conclusion is based on the observation that insertions of up to 30 bp do not significantly alter the hydroxyl radical cleavage pattern over the nucleosome dyad axis. In conjunction with our previous results, this suggests that the main determinants for translational positioning may be located between –146 and –76, in the central 70 bp of nucleosome B. Indeed, a comparison of the various positionings reported in the literature shows that this central core is included in the large majority of all translational frames described for the MMTV B nucleosome (12,16,27).

The rotational orientation of the double helix on the surface of nucleosome B is determined by the bendability of the nucleotide sequence (25). While constructing the insertion mutants we have tried to preserve the rotational phase found in the wild-type sequence by using alternating short tracts of G·C and A·T sequences. In fact, the insertions did not influence the rotational orientation of the upstream sequences, which, therefore, seems to be specified by sequence information upstream of the insertion point. As with the translational positioning, we know that sequences downstream of –146 are sufficient to determine the preferred rotational phase of nucleosomal DNA (26). Thus we conclude that the core sequence between –146 and –76 specifies not only the position of the nucleosome along the DNA but also rotational orientation of the MMTV promoter sequences and, therefore, accessibility of the HREs (17).

NF1 cannot bind to its cognate sequence in a nucleosome, no matter its rotational orientation

In previous experiments with reconstituted nucleosomes we have shown that the NF1 binding site is located 20 bp upstream of the proximal border of the core particle and is not accessible for NF1

binding (17). In these particles the two halves of the conserved palindrome which constitute the NF1 binding site were oriented with the major groove pointing to the interior of the nucleosome and we suggested that this was the reason for poor binding of NF1. Here we have tested this hypothesis by changing the orientation of the NF1 binding site by 105° and 180° through insertion of 3 and 5 bp respectively immediately upstream of the palindrome. The prediction was that those mutants with the major groove of the half palindromes exposed to the exterior of the nucleosomal particle should be bound by NF1. However, although the nucleosomes exhibited the predicted structure, no binding was observed to nucleosomes reconstituted with these constructions, indicating that exposing the major groove over the TGGA half sites is not sufficient to achieve stable interaction with NF1 on the surface of a nucleosome.

No NF1 binding was observed with reconstituted nucleosomes containing the NF1 binding site in the wild-type orientation but 10 bp closer to the proximal border of nucleosome B, a situation generated by a 10 bp insertion. Thus bringing the NF1 palindrome within 10 bp of the nucleosome border is insufficient to make the site accessible for NF1 binding. Similar results have been obtained with nucleosomes containing a NF1 binding site inserted within an artificial DNA bending sequence (19).

NF1 binds to its site on the edge of the nucleosome or in linker DNA even in the presence of histone H1

Different results were obtained when the NF1 site was moved to the edge of nucleosome B by inserting 30 bp. Nucleosomes reconstituted with promoters carrying this insertion exhibited a clear affinity for NF1, as demonstrated by band shift and exonuclease III protection experiments. Similarly, a promoter construction containing a 50 bp insertion, which moves the NF1 well into the linker DNA, also generated nucleosomes able to bind NF1. Therefore, we conclude that the affinity of NF1 for the MMTV promoter in mononucleosomes is determined by translational positioning of the nucleosome and is only possible when the NF1 palindrome reaches the border of the core particle. In mammary tumour cells carrying a chromosomally integrated copy of the MMTV promoter we have found a change in conformation of nucleosome B upon hormone induction, which is accompanied by binding of NF1 (13). Though we do not know the biochemical nature of this change, our present results suggest that a change in rotational orientation of the DNA is not enough to explain the hormone-induced binding of NF1 to the MMTV nucleosome.

One could argue that the ability of NF1 to bind to NF1 sites located in linker DNA was due to the absence in our reconstitution assays of linker histones, which would interact with the linker DNA and preclude binding. Such a repressive function of histone H1 has been postulated for the MMTV promoter, since hormonal induction leads to depletion of histone H1 from chromatin containing the promoter sequences (28). Though this is an attractive possibility, our preliminary experiments do not support such an explanation. Addition of stoichiometric amounts of histone H1 to the nucleosomes reconstituted with promoters carrying the 30 or 50 bp insertion generated a particle moving slower in acrylamide gels, which, therefore, likely contains the linker histone. We observed that even when the original population of nucleosome core particles was heterogeneous on acrylamide gels, the chromatosome particle containing histone H1 yielded a homogeneously migrating band, as if the linker histone imposed a dominant conformation on the mixture of core particles (29,30). This chromatosome band was clearly shifted upon addition of NF1 and yielded a retarded complex which likely contains NF1 along with core histones and histone H1. This assumption is based on the appearance of this retarded complex and on the fact that addition of NF1 generated neither core particles nor free DNA. Although a definitive proof awaits the demonstration of bound proteins by footprinting, we tentatively conclude that binding of histone H1 does not interfere with binding of NF1 to sites within the linker DNA.

The location of histone H1 in the nucleosomal array is controversial and histone H1 does not generate a footprint when bound to mononucleosomes. The classical view assumes that histone H1 is positioned outside the nucleosome core particle contacting the double helix at three points: the afferent and efferent linker DNA at the nucleosomal entry and exit points and at the pseudodyad (31–33). However, recent evidence suggests an alternative model, with the linker histones placed asymmetrically and inside the DNA superhelical path (34–35). In the most recent version of this asymmetrical model the linker histone is bound to the distal 5′-end of the nucleosome core particle (35). Our results would favour this latter model, as it may leave the NF1 site at the proximal 3′-linker of the nucleosome accessible for protein binding.

It is possible that the lack of an effect of histone H1 on NF1 binding to linker DNA is due to the use of mononucleosomes. A confirmation of our observation with the NF1 binding site included in the linker DNA between two adjacent nucleosomes is required. The evidence that hormone induction leads to deprivation of histone H1 over the MMTV promoter chromatin (28) suggests that linker histones may indeed be involved in restricting access to the MMTV promoter *in vivo*, possibly by influencing the higher order structure of chromatin. However, in *S.cerevisiae*, which supposedly lacks conventional linker histones, we observed tight control of MMTV transcription by steroid hormones (36).

A general rule for access to nucleosomally organized *cis*-elements?

The observation that the NF1 site is not accessible when included in a positioned nucleosome confirms results of *in vivo* footprinting experiments (13,14) and is consistent with our analysis of protein–DNA contacts. Using three different interference methods we have defined 23 contacts between NF1 and its binding site on the MMTV promoter: 13 contacts with base pairs (four guanines and nine thymines) through the major groove and 10 phosphate contacts. The position of these contacts on the double helix makes clear why the protein cannot bind its cognate site in the context of nucleosomes. The contacts map around the circumference of the double helix, which is almost completely embraced by the protein. This high number of contacts is not unexpected, since NF1 binds to its target sequence on free DNA with high affinity (22). Intimate contact with the double helix is not restricted to the few base pairs forming the conserved palindrome and cannot take place when the DNA interacts with core histones within the confines of a nucleosome. Not only will interaction with histones preclude binding of NF1 to the contacted phosphates, but it will also interfere with access to several of the contacted bases.

The binding behaviour of NF1 is in marked contrast to the behaviour of glucocorticoid and progesterone receptors, which can bind to their nucleosomally organized cognate sequences

provided their major grooves are exposed to the exterior of the particle (16,17). The explanation for this difference may reside in the very different kind of contacts made by the hormone receptors with the HREs. In contrast to NF1, hormone receptors bind DNA with low affinity and only contact the HREs at 12 positions, six on each half of the palindrome (7,23). More importantly, these contacts are clustered over a narrow sector of the helix circumference and the contacts with both halves of the palindromic HREs are on the same side of the double helix. Therefore, the relevant contact sites on the DNA are accessible from one side and could be contacted by receptors even when the opposite side of the double helix is occupied by core histones.

We would like to suggest that the hormone receptors and NF1 may be examples of DNA binding proteins with different abilities to recognize binding sites included within positioned nucleosomes. Proteins with high affinity for DNA are expected to embrace the double helix and contact bases and phosphates at many positions. These proteins, exemplified here by NF1, may be unable to recognize their binding sites in nucleosomes, no matter their rotational orientation. They may be able to interact with their cognate sites when they are located in linker DNA, although this statement must be confirmed with larger nucleosome arrays and in *in vivo* experiments. To this class of proteins may belong AP1 and other leucine zipper dimers, as well as NF-κB, the octamer transcription factors and other ubiquitous high affinity DNA binding proteins (see Beato and Eisfeld, this issue, ref. 37 for further discussion).

On the other hand, proteins such as the hormone receptors, which bind DNA with relatively low affinity, interact through only a few contacts with a narrow sector of the double helix and, thus, are able to recognize their cognate sequences within regular and positioned nucleosomes provided their rotational orientation permits access to the relevant major groove. No other DNA binding protein has been unambigously shown to belong to this class, which can bind to regular nucleosomes and act as initiators of regulatory events. Though some proteins, like the heat shock factor, can mount a regulatory response, they very often interact with sites located in nucleosome-free regions in the context of a preset chromatin (37). These initiators of chromatin remodelling occupy a higher position in the regulatory hierarchy, since their binding to DNA in chromatin is a prerequisite for high affinity DNA binding proteins to gain access to their cognate sequences. The mechanism by which initiators are able to remodel chromatin is unknown. One plausible mechanism is recruitment of one of the several chromatin remodelling complexes recently identified, such as the SWI–SNF complex (3) or NURF1 (39). In this respect, it is interesting to note that glucocorticoid receptor action seems to be compromised in the absence of components of the SWI–SNF complex (40,41). This complex has been suggested to facilitate dissociation of histone H2A–H2B dimers from nucleosomes (42) and we have recently shown that the MMTV promoter positioned on the surface of a tetramer of histones H3 and H4 is able to bind NF1 with relatively high affinity (C.Spangenberg, K.Eisfeld, K.Luger, T.E.Richmond, M.Truss and M.Beato, manuscript submitted).

ACKNOWLEDGEMENTS

We thank Axel Scholz and Bernhard Gross for cloning and preparing recombinant NF1, Alexander Schelbert for cloning some of the insertion mutants, Gerd Schlauderer (Giessen) for help with preparing the colour figures and Jörg Klug for advice on the manuscript. R.C. was a recipient of a long-term EMBO Fellowship. The experimental work has been supported by grants from the European Union, the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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