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Transcription Factor Access Is Mediated by Accurately Positioned Nucleosomes on the Mouse Mammary Tumor Virus Promoter

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A fragment of the mouse mammary tumor virus (MMTV) promoter was reconstituted from pure histones into a dinucleosome with uniquely positioned octamer cores. Core boundaries for the in vitro-assembled dinucleosome corresponded to the observed in vivo phasing pattern for long terminal repeat nucleosomes A and B. Nuclear factor 1 (NF1), a constituent of the MMTV transcription initiation complex, was excluded from the assembled dinucleosome, whereas the glucocorticoid receptor was able to bind. During transcription of MMTV in vivo, displacement of nucleosome B was necessary to permit assembly of the initiation complex. These results indicate that the nucleoprotein structure of the promoter can provide differential access to sequence-specific DNA-binding proteins and that active chromatin remodeling can occur during transcription activation.

The DNA in eucaryotic cells is continuously wrapped on a series of repeating histone octamer cores, giving rise to long polynucleosome arrays. In its path around the nucleosome, the DNA molecule is intimately associated with the histones. The influence that this organization may have on interactions between diffusible control proteins and their recognition sites on the DNA template is poorly understood (for reviews, see references 8, 15, 18, and 25). Regions of DNA hypersensitive to nucleolytic attack are often inferred to be nucleosome free, but it is not clear whether octamer cores are excluded from these regions during some particular period of nucleosome instability, such as replication, or whether preexisting cores can actually be displaced from the template. Furthermore, we now know of several examples for which the octamer cores can be quite precisely positioned, or phased, with regard to specific sequences. In these particular cases, the effect of nucleosome position on the binding of a given transcription factor is of potential significance.

Inducible genes present an obvious possibility to examine this issue directly. The yeast *PHO5* locus and the long terminal repeat (LTR) of mouse mammary tumor virus (MMTV) are two examples of inducible promoters whose chromatin structure has been well characterized (1, 31). The MMTV LTR reproducibly acquires a series of six positioned nucleosomes when introduced in mammalian cells. Although this phasing pattern was originally described with MMTV DNA sequences on highly amplified episomes (27), we have recently established that the same positioning is observed for integrated MMTV sequences (2, 31a). MMTV LTR sequences therefore include information specifying a reproducible nucleoprotein structure, although the mechanism(s) by which this chromatin pattern is acquired are not understood. Changes to this nucleoprotein structure occur quite rapidly, eliminating the elements of cell growth, differentiation, and division that are coincident with the establishment of many previously characterized alterations in chromatin structure.

Several proteins involved in hormone-dependent activation of the MMTV promoter have been characterized, including the steroid receptors (5, 37), which can act at a considerable distance from the site of transcription initiation, and two members of the immediate transcription initiation complex, nuclear factor 1 (NF1) and transcription factor IID (TFIID) (10, 11, 26). Given our detailed knowledge of both the chromatin structure and relevant transcription factors for the MMTV LTR, this promoter represents an interesting model with which to examine the impact of nucleoprotein structure on DNA-transcription factor interactions. It should be possible in this system to critically establish the effect, if any, of a specific nucleoprotein organization on the access and activity of specific DNA-binding proteins.

We therefore initiated efforts to reconstitute in vitro the putative chromatin structure of the MMTV LTR and to examine the impact of this structural organization on binding of the operative transcription factors. We have studied the assembly of histone octamer cores on a fragment of MMTV DNA containing the promoter and associated regulatory sequences, a region associated in vivo with two positioned nucleosomes, referred to as A and B (31). We find that octamer cores will position on this dinucleosome DNA fragment in vitro in a pattern consistent with the in vivo mapping experiments. These findings strongly support our previous conclusion that the unique pattern of chemical and nuclease sensitivity observed in vivo was the result of nucleosome phasing. We find that association of the promoter region sequences with positioned octamer cores A and B prevents the binding of NF1 to its cognate binding site at position −75 in the promoter. Therefore, the previously reported exclusion of NF1 from its binding site on LTR DNA (11) may be explained by the sequestration of the binding site by a positioned nucleosome. In contrast, the glucocorticoid receptor (GR), a protein with lower intrinsic affinity for DNA, is able to bind to its cognate site when the DNA is assembled in chromatin. We conclude that active remodeling of the MMTV nucleoprotein template occurs during transcription activation and that one consequence of this remodeling process is to permit access of at least one transcription factor that is excluded from noninduced promoter chromatin.

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MATERIALS AND METHODS

Cell lines and plasmids. Cell line 1361.5 is an NIH 3T3 transformant containing a multicopy bovine papillomavirus (BPV) episome (pm23) with the MMTV LTR driving the v-Ha-*ras* gene (11). Cell lines 1471.1 and 904.13 are BPV transformants of C127 cells. Cells were maintained in Dulbecco modified Eagle medium containing 10% fetal calf serum. Hormone induction of cell cultures was carried out by addition of the specified concentration of dexamethasone to the culture medium for the indicated time periods.

Plasmid pM50 (10) contains a fragment of the C3HS MMTV LTR (12) from the *Hae*III site at position −223 to the *Hpa*II site at position +107 inserted at *Eco*RI-*Bam*HI in pSP65 (Promega Biotec). Plasmid pC124 contains a mutated LTR fragment −221 to +107, with the *Hae*III site at −223 converted to an *Apa*I site at −221 and an *Eco*RI* site at −75 converted to an *Eco*RI site, cloned into pGEM4 (22, 34a). Plasmid pNBOA was constructed by the synthesis of oligonucleotides corresponding to −75 to +107 of the MMTV LTR, which were ligated to a fragment corresponding to −221 to −75 of the LTR. This fragment was obtained from plasmid pC124. The oligonucleotides were identical to the wild-type C3HS sequence except that sites for *Bcl*I (−48), *Bgl*I (−16), and *Sac*II (+65) were inserted without changing the length of the nucleosomal linker or the length of the A-B fragment in pC124. DNA fragments were uniquely end labeled with [^{32}P]ATP (6,000 Ci/mmol; New England Nuclear), using T4 polynucleotide kinase as described previously (3).

In vivo chromatin analysis. Nuclei were prepared from a monolayer of 10^8 cells as described previously (11). Briefly, cells were resuspended in 10 ml of homogenization buffer (10 mM Tris hydrochloride [pH 7.4], 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.1 mM ethyleneglycol-bis-*N*,*N*′-tetraacetic acid [EGTA], 0.2% Nonidet P-40, 5% sucrose), allowed to stand for 3 min on ice, and homogenized by 10 strokes of a glass Dounce A pestle. Nuclei were sedimented through a 10% sucrose cushion, and the nuclear pellet was recovered and washed in 10 ml of wash buffer (10 mM Tris hydrochloride [pH 7.4], 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine). After collection by centrifugation at 800 × *g* for 4 min at 0°C, washed nuclei were resuspended in enzyme digestion buffer (10 mM Tris hydrochloride) [pH 7.4], 15 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol) at 5 × 10^8 nuclei per ml. The appropriate restriction enzyme was included in the digestion buffer (between 1,000 and 4,000 U/ml), and aliquots of 2.5 × 10^7 nuclei were digested at 30°C for 15 min. The digestion was stopped by addition of 10 volumes of 10 mM Tris hydrochloride (pH 7.6)–10 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–100 μg of proteinase K per ml and incubated for 2 h at 37°C. Nucleic acids were purified by extractions with phenol and chloroform and precipitation in ethanol. Five micrograms of purified DNA was subjected to secondary limit digestion with the appropriate restriction enzyme for indirect end-labeling analysis and electrophoresed on a standard 5% DNA sequencing gel. After electrophoresis, DNA fragments were transferred to a nylon membrane (ICN) in a specially adapted vertical electrotransfer apparatus as described previously (11). Prehybridization and hybridization with appropriate indirect end-labeled probes were performed as described previously (11).

Preparation of core histones and core particles. Murine cell lines 1361.5 and 1471.1 were used to prepare core histones by the method of Germond et al. (17) as modified by Dunn and Griffith (14). Briefly, cells were lysed with detergent and nuclei were purified prior to washing in buffer A (20 mM $NaPO_4$ [pH 6.6], 1 mM EDTA, 25 mM sodium acetate, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol) containing 0.8 M NaCl to remove histone H1. Following centrifugation, the H1-depleted chromatin was resuspended in buffer A containing 2.0 M NaCl, sonicated, and then passed over hydroxyapatite (equilibrated in the same buffer) to remove acidic proteins and nucleic acids. Individual protein preparations were checked by polyacrylamide gel electrophoresis and then stored in this buffer at 1 mg/ml over liquid N_2.

Core particles from murine cell line 904.13 were prepared by limited micrococcal nuclease digestion of nuclei and sucrose gradient purification of mononucleosomes as described by Ausio et al. (4). Individual protein preparations were checked by polyacrylamide gel electrophoresis and titrated in reconstitution experiments prior to being stored at 1 mg/ml at 4°C.

In vitro assembly of nucleosomes. Reconstitutions were performed by modifications of the procedures of Dunn and Griffith (14) and Lorch et al. (23). Core histones were deposited on DNA by mixing ^{32}P-end-labeled DNA (40 to 80 ng) in 2.0 M NaCl with a range of histone concentrations in 2.0 M NaCl and then diluting stepwise to 1.0, 0.5, 0.25, 0.125, and 0.1 M with a buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 1 mM EDTA, and 0.5 mM dithiothreitol) in 10-min intervals at room temperature.

In a standard reaction, 4 μg of core particles was incubated with 40 ng of end-labeled DNA in 0.8 M NaCl, and the salt was reduced to a final concentration of 100 mM NaCl with a buffer (50 mM Tris [pH 8.0], 12 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10% glycerol) overnight. Nucleosomes were analyzed by gel electrophoresis on 5% polyacrylamide gels with low bis/acrylamide ratios (1:50 or 1:37.5) in 36 mM Tris–36 mM boric acid–1 mM EDTA. Gels were dried and then subjected to autoradiography. Reconstituted chromatin by both procedures gave indistinguishable results.

Restriction endonuclease access in vitro. Reconstituted dinucleosomes were adjusted to 10 mM $MgCl_2$, specified restriction endonuclease were added to 100 or 1,000 U/ml, and the reaction was allowed to proceed for 1 h at 25 or 37°C. In the case of *Bcl*I, the digestion was at 50°C for 1 h. The reconstituted chromatin was stable under all conditions examined (see Fig. 3E and 5). Reactions were stopped with SDS (0.1%) and subjected to phenol-chloroform extractions and ethanol precipitation. Purified DNA was analyzed on denaturing polyacrylamide gels as described in Cordingley et al. (11). For some *Bcl*I digestions, the samples were analyzed on nondenaturing gels without prior purification of the DNA.

NF1 exclusion from reconstituted nucleosomes. Nucleosomes reconstituted as described above were incubated with NF1 that was purified as described by Cordingley and Hager (10), with the modification that the final NaCl concentration was maintained at 100 mM and binding was at 25°C for 30 min. Exonuclease III (ExoIII)-resistant boundaries (36) were located as described previously (11) with ExoIII (1,000 to 10,000 U/ml). After 15 min of digestion at 30°C, reactions were stopped with 1% SDS and 10 mM EDTA and were then purified and analyzed on denaturing sequencing gels as described above.

Gel shift assays for NF1 and GR. Free DNA and dinucleosomes reconstituted as described above (2 to 4 ng) were incubated with purified NF1 (1 μg) and GR fragment 440-525

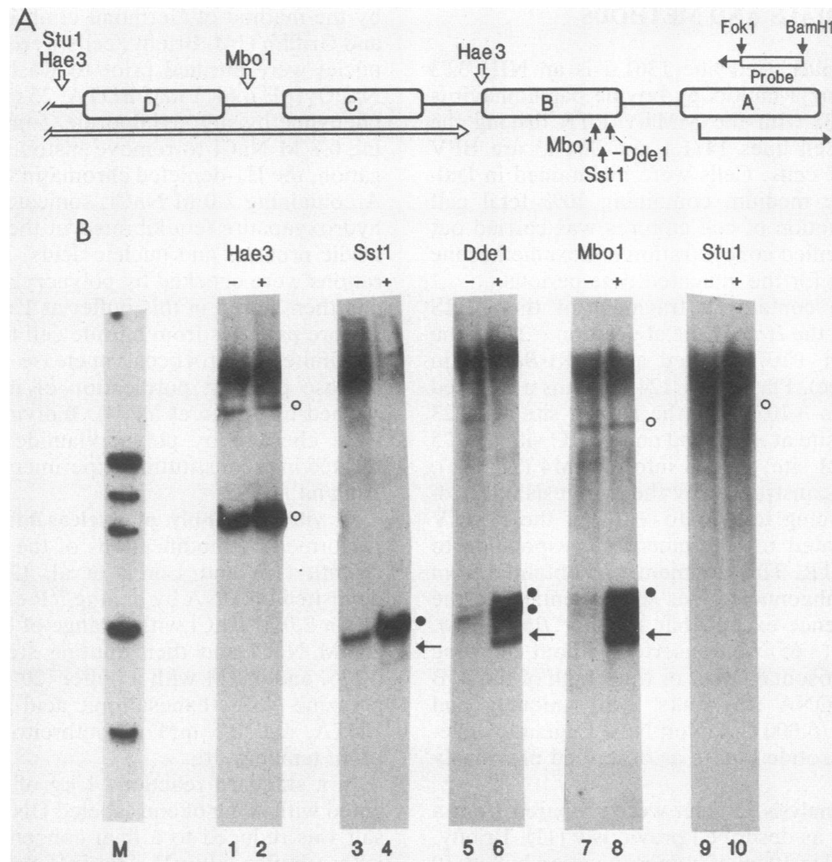

FIG. 1. Enzyme accessibility of sequences in MMTV LTR chromatin. The susceptibility of sequences in LTR minichromosome nucleoprotein to digestion was determined for a variety of restriction endonucleases. (A) Schematic representation of the predicted nucleosome positions for the MMTV LTR (31) and restriction enzyme site positions. The horizontal open arrow denotes the end of the LTR open reading frame (12). (B) Products of nuclear digestions for a series of enzymes (indicated above the lanes). Nuclear isolations, enzyme digestions, and indirect end-labeling analysis were performed as described in Materials and Methods. Lanes 1, 3, 5, 7, and 9 represent the products for digestions of nuclei from untreated cells, and lanes 2, 4, 6, 8, and 10 represent those from hormone-stimulated cells. Open circles represent restriction fragments whose relative abundance is unaffected by hormone treatment, and closed circles represent those whose concentration is hormone dependent. The arrows in lanes 4, 6, and 8 mark the NF1 ExoIII boundary (see text). Lane M contains a *Hin*dIII digest of lambda DNA as markers.

(0.1 μg) in a final concentration of 100 mM NaCl for 20 min at 25°C and then loaded onto nondenaturing polyacrylamide gels as outlined earlier (10, 20, 23). Following electrophoresis gels were dried and then subjected to autoradiography.

RESULTS

In vivo enzyme sensitivity of LTR phased array. Previous experiments demonstrated that the MMTV LTR was organized in six phased nucleosomes and that the second (or B) nucleosome was massively altered in structure during hormone stimulation (19, 31). To permit a high-resolution comparison of in vivo chromatin and in vitro-reconstituted polynucleosomes, we carried out a detailed analysis of MMTV LTR chromatin sensitivity to specific restriction endonucleases. This analysis was performed on cells harboring high-copy-number chimeras that contain the MMTV promoter and various reporter genes. This BPV-MMTV model system and nucleosome positioning studies have been extensively described (2, 19, 27). The results of experiments performed with cell line 1361.5 (an NIH 3T3 cell harboring the pm23 chimera; 11) are presented in Fig. 1. Nuclei from either hormone-treated or control cells were isolated and treated with a variety of restriction endonucleases to determine the sensitivity of chromatin-organized MMTV promoter DNA to nucleolytic attack at a variety of positions. In some cases, more than one site for a given enzyme can be monitored as a partial digestion product. Positions of the various enzymes are depicted schematically in Fig. 1A in relation to the putative nucleosome positions (31). For *Hae*III, both the site in the linker region between nucleosomes D and E and the site at the left edge of nucleosome B are sensitive to attack, and the availability of both sites is unaffected by hormone treatment. The sensitivity to *Stu*I, whose site is also associated with a putative linker region, is also hormone independent. For enzymes whose recognition sites are located in the core region of nucleosome B, however, availability of the sites is strongly hormone dependent. This includes three enzymes, *Mbo*I, *Dde*I (two sites), and *Sst*I. The analysis with enzyme *Mbo*I is particularly useful in this regard, since one recognition site is located in the linker between nucleosomes C and D, and the second site is on the core of nucleosome B. As with the other linker-associated sites, the upstream *Mbo*I site is available

and nonresponsive to hormone treatment (open circle in Fig. 2B), while the downstream *Mbo*I site is completely hormone dependent (closed circle). Sensitivity to attack by this enzyme therefore provides a useful internal control. For the enzymes *Sst*I, *Dde*I, and *Mbo*I, a common band (indicated by the arrows) appears in the digests below the actual restriction fragments (indicated by the closed circles). This band corresponds to the hormone-dependent loading of NF1 and results from endogenous exonucleolytic attack of the respective DNA fragments (11).

These results are consistent with the earlier DNase I and methidiumpropyl-EDTA (MPE) hypersensitivity experiments (31); the only enzyme sites whose sensitivity is affected by hormone treatment are located on the core of nucleosome B. The results are also in agreement with the suggested nucleosome positions derived from micrococcal nuclease and MPE sensitivity analysis. Enzyme sites located in linker regions are open, whereas sites located on a putative octamer core region are accessible only when a hormone-dependent displacement event has occurred. The restriction enzyme access experiments outlined above entirely support the concept that nucleosomes are specifically positioned across the LTR as previously suggested and are consistent with a specific and massive structural alteration unique to the core region of nucleosome B. Second, they provide high-resolution accessibility landmarks with which to compare experiments on in vitro-reconstituted chromatin.

In vitro assembly of nucleosomes on LTR DNA. To compare the putative in vivo nucleosome array with an authentic polynucleosome ladder, we assembled octamer cores on MMTV DNA in vitro by the high-salt dilution technique (21, 23), utilizing MMTV DNA fragments isolated from plasmids and either purified histone H2A, H2B, H3, and H4 fractions or core particles purified from NIH 3T3 and C127 cells (4, 14, 17). The association of histones with DNA was monitored initially by the gel retardation assay. Specific histone-DNA complexes could be detected as a single, retarded band when octamer cores were assembled on a 360-bp DNA fragment containing sequences from −223 to +107 of the MMTV LTR, a region encompassing both nucleosomes A and B (Fig. 2A and 3E). This experiment indicates that assembly resulted in a monodisperse pool of nucleosomes; as we demonstrate below, this population is composed uniquely of disomic structures.

Positioning of nucleosome boundaries in vitro. Nucleosome positioning is commonly studied by the detection of boundaries resistant to the progress of ExoIII or by the presence of 10-nucleotide DNase I ladders that result from rotational positioning of DNA on the octamer core surface (33). Digestion of the in vitro-assembled A-B dinucleosome with ExoIII resulted in the appearance of three distinct ExoIII stops near the 5′ end of the DNA fragment (Fig. 2B, lanes 3 and 4); these blocks were not observed with pure DNA (lanes 1 and 2). Each of these ExoIII barriers (−221, −213, and −205; Fig. 3F) was within the error of measurement for the left boundary of nucleosome B determined in vivo (31). The multiple stops could result either from unique ExoIII blocks on a disome population heterogenous with respect to nucleosome B position or from ExoIII readthrough on a uniquely positioned nucleosome.

To further analyze the position of cores on the assembled nucleosomes, restriction enzyme sensitivity analysis was performed. This approach permits a direct comparison between the structures assembled in vitro and the putative in vivo polynucleosome array. A schematic of the DNA fragments used in these studies, with pertinent restriction en-

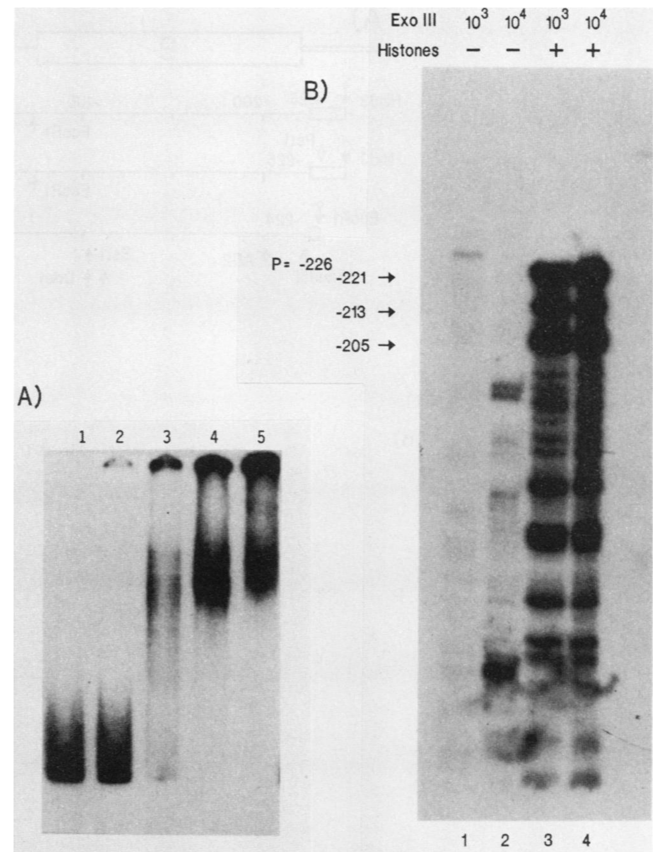

FIG. 2. Assembly of the A-B dinucleosome in vitro and determination of 5′ boundary. (A) Gel retardation analysis of dinucleosome. Nucleosomes were reconstituted as described in Materials and Methods and analyzed by polyacrylamide gel electrophoresis on nondenaturing gels. Lanes: 1, DNA without histones; 2, DNA with 0.1 μg of histones; 3, DNA with 0.2 μg of histones; 4, DNA with 0.5 μg of histones; 5, DNA with 1.0 μg of histones. (B) Determination of dinucleosome 5′ boundary by ExoIII digestion. The 360-bp fragment from pM50 was end labeled as described in Materials and Methods (see Fig. 3A) and assembled as chromatin as described above. Digestion of free DNA (lanes 1 and 2) and chromatin (lanes 3 and 4) with 10^3 (lanes 1 and 3) or 10^4 (lanes 2 and 4) U of ExoIII was for 30 min at 25°C. Following digestion, DNA was purified and analyzed on denaturing sequencing gels.

zyme sites and the in vivo boundaries for the dinucleosome, are indicated in Fig. 3A. In Fig. 3B, sensitivity of the in vitro dinucleosomes to enzymes active in the B nucleosome region is presented. In all cases, enzymes whose sites were predicted in vitro to reside on the octamer cores, *Dde*I (lanes 1 and 2), *Sst*I (lanes 3 and 4), *Eco*RI (lanes 5 and 6), *Afl*II (lanes 7 and 8), and *Sau*96I (lanes 9 and 10), were sensitive to histone deposition in vitro. *Pst*I, however (lanes 11 and 12), was able to attack its site even after assembly of cores on the DNA. The *Pst*I site is located in the same position as the accessible *Hae*III site in vivo. The positions of *Sau*96I and *Pst*I at −220 and −225 provide an opportunity to resolve the issue of multiple ExoIII stops. The lack of cleavage by *Sau*96I (−220) suggests that the second (−213) and third (−205) ExoIII blocks represent readthrough digestion products. The appropriate assignment for the 5′ boundary of nucleosome B would then be the first ExoIII stop at −221. This position is in good agreement with the −228 position inferred from the in vivo experiments (Fig. 3F).

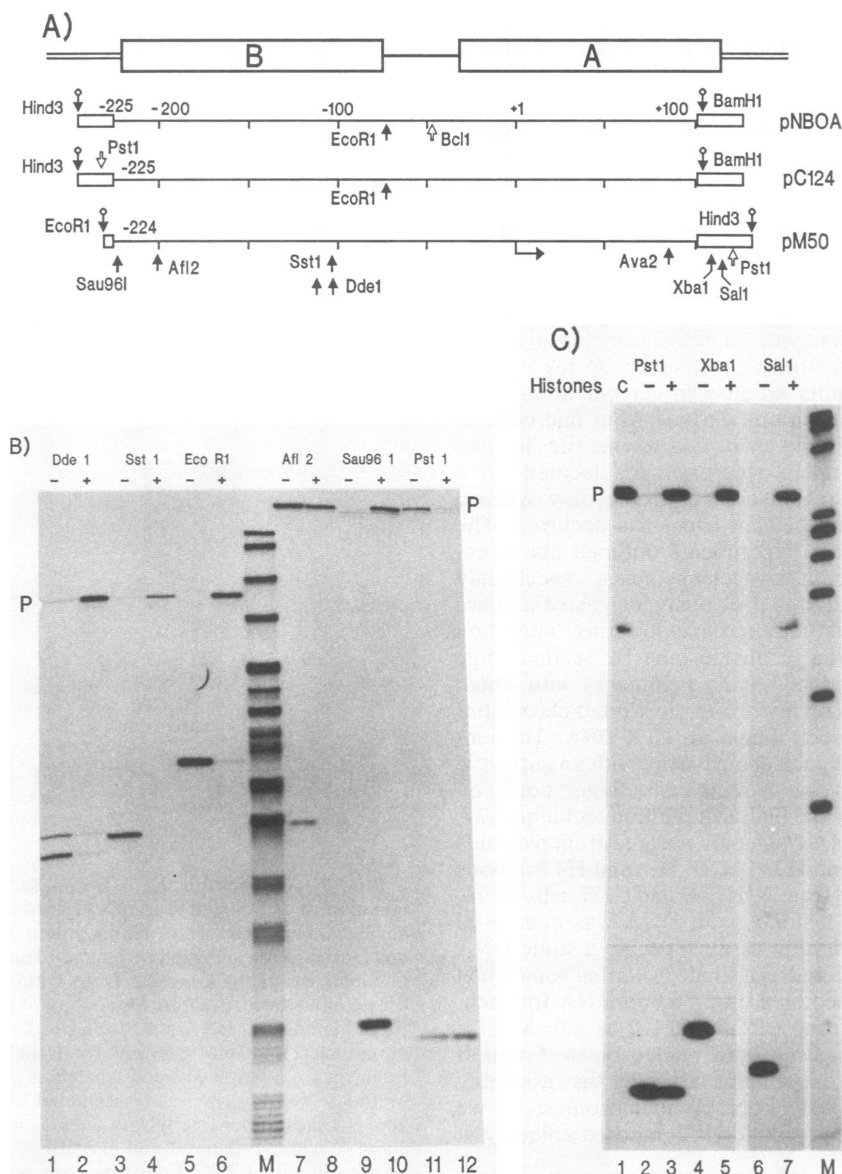

FIG. 3. Enzyme accessibility of sequences in the reconstituted A-B dinucleosome. (A) Schematic representation of the molecules used for reconstitution experiments. Pertinent restriction enzyme sites and in vivo boundaries of nucleosomes A and B are shown. Small open boxes at the end of the nucleosomes represent polylinker sequences present in the constructions. Fragments were liberated by *Hin*dIII-*Bam*HI cleavage for pNBOA and pC124 or *Hin*dIII-*Eco*RI digestion for pM50. Fragments were labeled at a single terminus prior to the second cleavage. Solid vertical arrows indicate sites predicted to be within core boundaries, and open vertical arrows indicate sites predicted to be outside core boundaries. (B) Accessibility of sequences in the nucleosome B region. Octamer cores were assembled on the A-B-region DNA fragment (end labeled at the *Hin*dIII site) purified from plasmid pC124 as described in Materials and Methods and the legend to Fig. 2. $MgCl_2$ was added to 10 mM, the indicated restriction enzymes were introduced, and digestion was allowed to proceed. Lanes 1, 3, 5, 7, 9, and 11 contained pure DNA and no histones (−). P, Position of the parental, uncleaved fragment. After digestion, reaction products were applied directly to 8% acrylamide sequencing gels and electrophoresed, and the resolved fragments were detected by autoradiography. Samples in lanes 1 to 6 and M (marker) were run into the gel 4 h prior to loading of samples in lanes 7 to 12. Lane M contained *Msp*I-digested pBR322 DNA as a marker. (C) Accessibility of sequences in the nucleosome A region. The dinucleosome was assembled on the A-B-region DNA fragment (end labeled at the *Hin*dIII site) purified from plasmid pM50 as described in Materials and Methods and the legend to Fig. 2. Enzyme digestion and analysis were performed as described above, and enzymes are indicated above the relevant lanes. Lanes 2, 4, and 6 contained pure DNA and no histones (−). Lane C contained the input DNA not subjected to any enzyme digest, and lane M contained *Hae*III-digested φX174 DNA as a marker. (D) Lack of nonspecific inhibition by histones. Free and reconstituted DNA from pC124 were mixed such that alternate ends of the DNA were labeled and then subjected to enzyme digestion and analysis as for panels B and C. The schematic adjacent to the autoradiogram indicates that lanes 1 and 2 contained reconstituted DNA labeled at the *Bam*HI terminus (*) and free DNA labeled at the *Hin*dIII end (*), while lanes 3 and 4 contained reconstituted DNA labeled at the *Hin*dIII end (*) and free DNA labeled at the *Bam*HI terminus (*). Digestion with *Dde*I produces significant cleavage of the free DNA, as indicated by the preeminence of the lower doublet versus the upper doublet derived from the reconstituted DNA. When the labelings of the free and reconstituted samples are reversed, the predicted cleavage differences are again observed in lane 3. Cleavages by *Afl*II (lane 2) and *Ava*II (lane 4) are efficient on naked DNA but not on the reconstituted DNA, and cleavage of the free DNA is not inhibited by the presence of core histones. (E) Susceptibility of linker region DNA to attack. Octamer cores were assembled on the A-B-region DNA fragment (end labeled at the *Bam*HI site [nucleosome A]) purified from

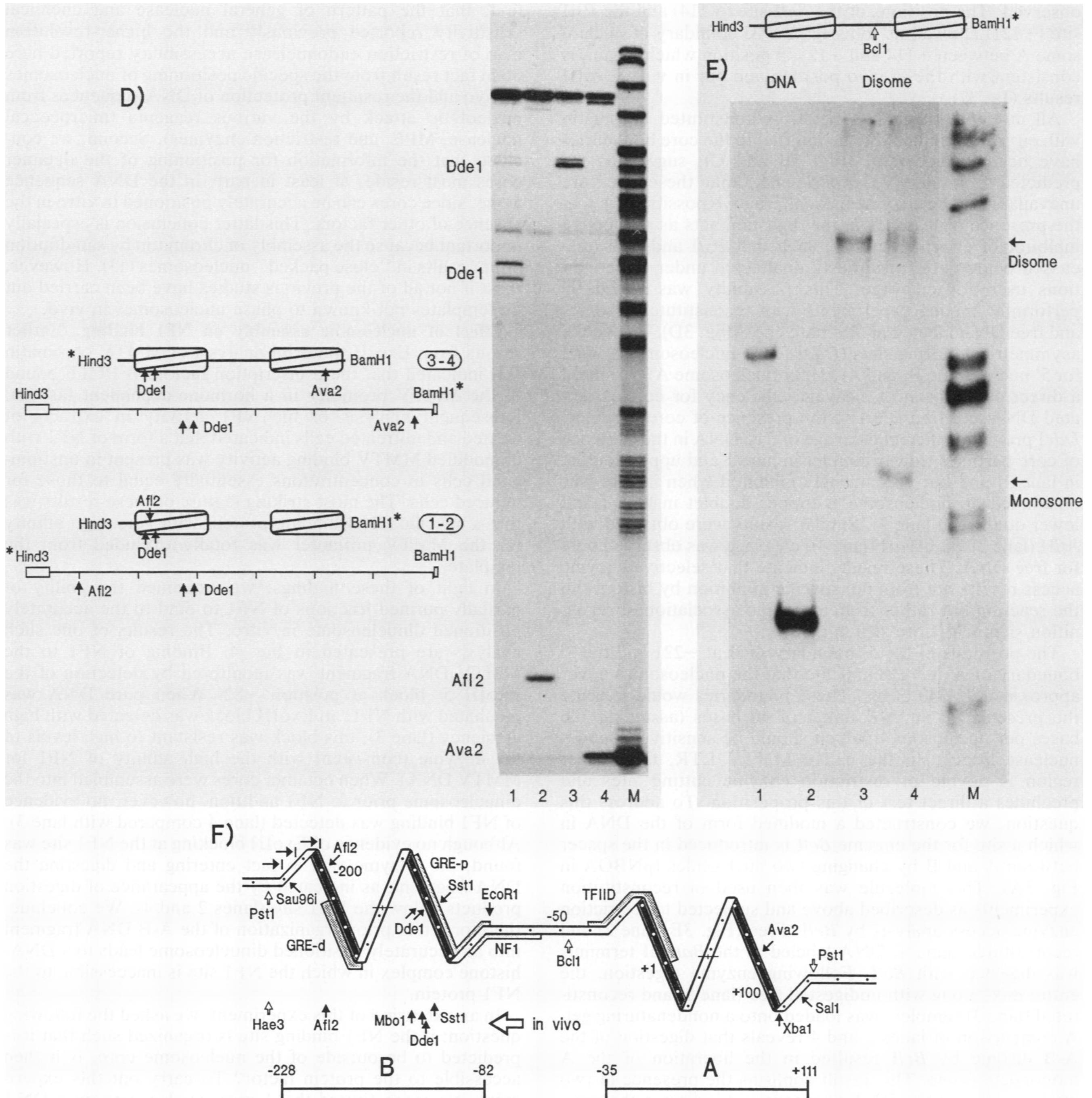

FIG. 3—*Continued.*
plasmid pNBOA as described in Materials and Methods. Free DNA (lanes 1 and 2) and reconstituted nucleosomes (lanes 3 and 4) were digested with *Bcl*I (lanes 2 and 4) and then analyzed on a nondenaturing gel. Lane M contained *Hae*III-digested φX174 DNA as a marker. Migration of the A mononucleosome relative to the A-B dinucleosome is indicated at the right. (F) Summary of in vitro and in vivo nucleosome positions. The proposed disposition of core and linker region DNA for the A-B disome and the enzyme accessibility data are diagrammed. In vivo enzyme sensitivities (this report) and in vivo core positions (Richard-Foy and Hager [31]) are displayed below the schematic for comparison. Solid vertical arrows indicate hormone dependence of access, and open vertical arrows indicate hormone independence. The ExoIII boundaries (Fig. 2B) are indicated by the horizontal arrows and vertical bars. Inferred positions of dyad symmetry for each of the cores are designated (-+-). NF1, TFIID, distal GR (GRE-d) and proximal GR (GRE-p) binding sites are indicated by cross-hatched boxes.

In Fig. 3C, the results of enzyme access analysis are presented for enzymes with sites located in the nucleosome A region. For *Xba*I (lanes 4 and 5) and *Sal*I (lanes 6 and 7), both of which have recognition sites located in the core region of nucleosome A, the ability to digest the template was inhibited by prior assembly of the nucleosome. With *Pst*I, however, whose site is predicted to be external to the A core region, a significant degree of enzyme cutting was

observed. The positions of the *Sal*I site (+114) and the *Pst*I site (+121) allowed us to assign the 3′ boundary of nucleosome A between +114 and +121, a position which, again, is consistent with the in vivo position and our in vitro ExoIII results (1a, 31).

All attempts to cleave in vitro-reconstituted chromatin with enzymes predicted to lie internal to the core boundaries have been unsuccessful (Fig. 3B and C), suggesting (as predicted from in vivo experiments) that these sites are unavailable to the enzymes. An alternative possibility is that the presence of histones in the digestion acts as a selective inhibitor of enzyme activity, such that *Pst*I and *Bcl*I (discussed below) are fortuitously unaffected under the conditions used for cleavage. This possibility was tested by performing mixing experiments with reconstituted disomes and free DNA labeled at alternate ends (Fig. 3D). The use of asymmetrical cutting sites (*Dde*I for 3′ nucleosome B, *Afl*II for 5′ nucleosome B, and *Ava*II for nucleosome A) permitted a direct comparison of cleavage efficiency for core-associated DNA and free DNA in the presence of core particles. *Dde*I provided efficient cleavage of free DNA in the presence of core particles (lower doublet in lane 1 and upper doublet in lane 3) and but was strongly inhibited when its site was positioned on nucleosome B (upper doublet in lane 1 and lower doublet in lane 3). Similar results were obtained with *Afl*II (lane 2) and *Ava*II (lane 4); cleavage was observed only for free DNA. These results indicate that selective enzyme access results not from nonspecific inhibition by histones in the reaction but rather from a specific association of recognition sites with core structures.

The positions of the 5′ boundary of B at −221 and the 3′ boundary of A at +118 indicate that the nucleosomes cover approximately 340 bases. These boundaries would indicate the presence of an A-B linker of 40 bases (assuming 146 bases per nucleosome), which should be sensitive to endonuclease attack. In the native MMTV LTR, this spacer region is devoid of restriction enzyme cutting sites and precludes a direct test of this proposition. To address this question, we constructed a modified form of the DNA in which a site for the enzyme *Bcl*I is introduced in the spacer between A and B by changing two nucleotides (pNBOA in Fig. 3A). This molecule was then used in reconstitution experiments as described above and subjected to restriction enzyme access analysis by *Bcl*I. Free (Fig. 3E, lane 2) and reconstituted (lane 4) DNA labeled at the *Bam*HI terminus was digested with *Bcl*I. Following enzyme digestion, the entire mix, along with undigested free (lane 1) and reconstituted (lane 3) samples, was loaded onto a nondenaturing gel. A comparison of lanes 2 and 4 reveals that digestion of the A-B disome by *Bcl*I resulted in the liberation of the A mononucleosome. This result confirms the presence of two nucleosomes on the DNA fragment and indicates the presence of an enzyme-accessible region equivalent to the linker region observed in vivo.

The data presented in Fig. 2 and 3B to E are summarized in Fig. 3F. The boundaries for nucleosomes B and A obtained in vitro are identical within experimental error to those described previously in vivo. The in vitro results demonstrate that enzymes whose recognition sites are predicted from the in vivo analysis to be located on the core region of either nucleosome A or B are inhibited by chromatin assembly, while those sites predicted to be external to the core regions show a significant degree of enzyme cleavage. In addition, the insertion of a novel enzyme cutting site into the presumptive A-B linker region results in a site that is accessible in in vitro-reconstituted chromatin. We conclude, first, that the pattern of general nuclease and chemical sensitivity reported previously and the higher-resolution map of restriction endonuclease accessibility reported here do in fact result from the specific positioning of nucleosomes in vivo and the resultant protection of DNA sequences from nucleolytic attack by the various reagents (micrococcal nuclease, MPE, and restriction enzymes). Second, we conclude that the information for positioning of the octamer cores must reside, at least in part, in the DNA sequence alone, since cores can be accurately positioned in vitro in the absence of other factors. This latter conclusion is especially important because the assembly of chromatin by salt dilution often results in "close packed" nucleosomes (13). However, most if not all of the previous studies have been carried out on templates not known to phase nucleosomes in vivo.

Effect of nucleosome assembly on NF1 binding. Earlier results from ExoIII footprint analysis of MMTV chromatin (11) indicated that the transcription factor NF1/CTF bound to the MMTV promoter in a hormone-dependent fashion. Subsequent analysis of the NF1 activity in extracts of treated and untreated cells indicated that a form of NF1 with unmodified MMTV binding activity was present in unstimulated cells in concentrations essentially equal to those for induced cells. The most striking feature of these results was that a specific DNA-binding protein with very high affinity for the MMTV promoter was totally excluded from the template.

In light of these findings, we examined the ability of partially purified fractions of NF1 to bind to the accurately positioned dinucleosome in vitro. The results of one such analysis are presented in Fig. 4. Binding of NF1 to the MMTV DNA fragment was monitored by detection of the ExoIII 5′ block at position −82. When pure DNA was incubated with NF1, an ExoIII block was detected with high efficiency (lane 3); this block was resistant to high levels of the enzyme (consistent with the high affinity of NF1 for MMTV DNA). When octamer cores were assembled into the dinucleosome prior to NF1 addition, however, no evidence of NF1 binding was detected (lane 4 compared with lane 3). Although no evidence of ExoIII blocking at the NF1 site was found, the enzyme was in fact entering and digesting the DNA fragment, as indicated by the appearance of digestion products below the NF1 site (lanes 2 and 4). We conclude, therefore, that prior organization of the A-B DNA fragment into an accurately positioned dinucleosome leads to a DNA-histone complex in which the NF1 site is inaccessible to the NF1 protein.

In an extension of this experiment, we asked the following question: if the NF1 binding site is organized such that it is predicted to be outside of the nucleosome core, is it then accessible to the protein factor? To carry out this experiment, we reconstituted the A mononucleosome on a DNA fragment that contained the NF1 binding site in addition to the DNA normally incorporated into the A mononucleosome. In this way, we are attempting to partially reproduce the target for NF1 as it is predicted to occur in vivo when, upon hormone treatment, nucleosome B is displaced by nucleosome A is unaffected. The results of these preliminary experiments indicate that NF1 is able to bind to its recognition site in vitro when the adjacent sequences are assembled in chromatin (data not shown). Taken in conjunction with the previous results, these data further establish that the accurate positioning of octamer cores over the MMTV LTR, as determined in vivo, is sufficient to prevent the binding of NF1.

The GR but not NF1 can bind to chromatin. Hormone-

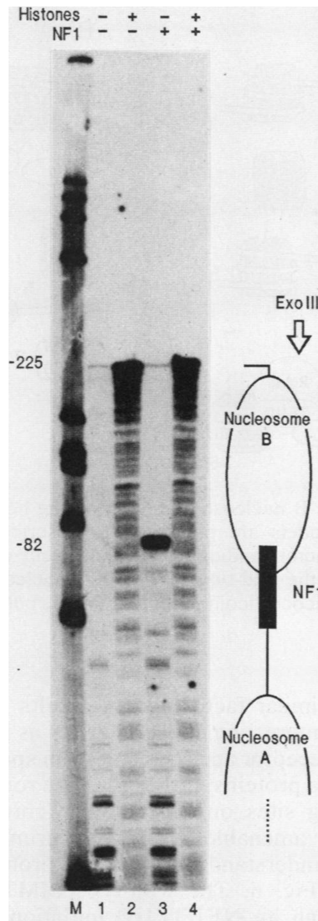

FIG. 4. Exclusion of NF1 from binding by nucleosome assembly. Nucleosomes were reconstituted as described above and then incubated with purified NF1 for 30 min at room temperature (25°C), followed by the addition of ExoIII as described previously (11). Lane M contained *Hae*III-digested ϕX174 DNA as a marker. The schematic at the right positions the site of the NF1 binding site relative to the core boundaries determined in vivo and in vitro. Lanes 1 and 3 contain free DNA; lanes 2 and 4 contain reconstituted nucleosomes. Products from reactions containing NF1 are displayed in lanes 3 and 4.

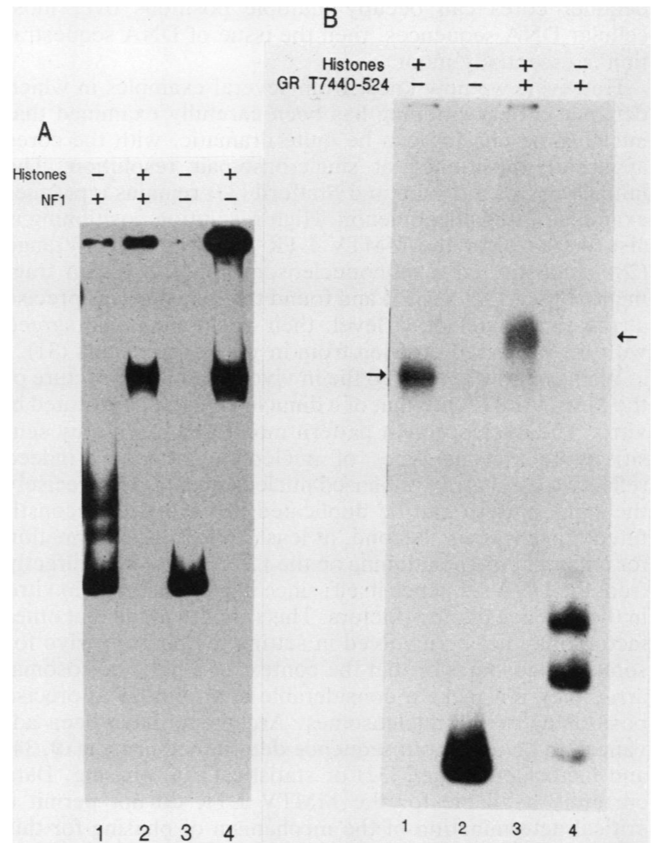

FIG. 5. Ability of GR but not NF1 to form a complex with the A-B dinucleosome. (A) NF1 is unable to bind chromatin in vitro. The assembly of chromatin, binding of NF1, and gel analysis were as described in Materials and Methods. Lanes: 1 and 3, free DNA; 2 and 4, reconstituted chromatin. NF1 was included in reactions displayed in lanes 1 and 2. (B) The DNA-binding domain of the rat GR binds both free DNA and chromatin. Experimental conditions were as described above except that purified T7(440-525), the DNA-binding domain of the rat GR, was added to either free DNA (lane 4) or chromatin (lane 3). Addition of the GR to chromatin resulted in a tertiary complex of DNA-octamer cores-GR (compare lanes 1 and 3) that has mobility different from that of the reconstituted DNA alone (lane 1), the free DNA (lane 2), or the receptor–free-DNA complex (lane 4).

dependent induction of transcription from the MMTV LTR in vivo is initiated by the GR. The receptor must therefore be able to interact with its site organized as chromatin. We examined NF1 and receptor binding to chromatin directly in a gel shift assay utilizing free DNA and the reconstituted A-B disome (Fig. 5A). The addition of NF1 to free DNA harboring the MMTV −75 NF1 site resulted in the formation of retarded complexes (compare lanes 1 and 3). When NF1 was added to the disome, however, no evidence of interaction was obtained (lanes 2 and 4). These results constitute independent evidence that NF1 is unable to interact with its site in chromatin.

To examine the interaction of purified receptor with the A-B disome, we utilized the DNA-binding domain of the rat GR, expressed in bacteria (20). In contrast to the results obtained with NF1, when the GR DNA-binding domain was allowed to interact with the disome (Fig. 5B), a tertiary complex of DNA-octamer cores-GR was formed (lane 3) that had a mobility slower than that of either the reconstituted disome alone (lane 1), free DNA (lane 2), or the receptor–free-DNA complex (lane 4). Thus, the access of two purified transcription factors to their sites in the in vitro-assembled chromatin paralleled remarkably well the inferred behavior of these molecules in the nucleus.

DISCUSSION

The organization of eucaryotic DNA into repeating nucleosome structures solves a major problem for the cell. By repeated condensation of these arrays into higher-order compacted assemblies, the very large amounts of DNA present in these cells can be stored in relatively small volumes. When the nucleosome paradigm was first established, it was apparent that the potential exists in this structure for the exclusion of a subset of DNA sequences from access by sequence-specific DNA-binding proteins. The general importance of this issue is related to the question of sequence-specific positioning of nucleosomes. If

octamer cores can occupy multiple positions over most cellular DNA sequences, then the issue of DNA sequestration is essentially moot.

However, we now know from several examples in which octamer core positioning has been carefully examined that nucleosome phasing can be quite dramatic, with the cores apparently positioned at single-base-pair resolution. The initial work of Simpson and Stafford (33) remains a premier example of this phenomenon. High-resolution positioning is also the case for the MMTV LTR. Perlmann and Wrange (28) reconstituted a mononucleosome on a B-region fragment of the MMTV LTR and found that phasing was precise at the single-nucleotide level; their positioning also agreed with the predicted position from in vivo experiments (31).

We have now compared the in vivo chromatin structure of the MMTV LTR with that of a dinucleosome reconstituted in vitro. The nucleoprotein pattern monitored in vivo by sensitivity to various types of nucleolytic attack is indeed reflective of an array of phased nucleosomes, since precisely the same pattern can be duplicated in vitro with reconstituted nucleosomes. Second, at least part of the information for octamer core positioning on the LTR must derive directly from the DNA sequence itself, since phasing is found in vitro in the absence of other factors. This is not to argue that other factors may not be involved in setting the phase in vivo for some nucleosomes or that the context of a polynucleosomal array may not make a considerable contribution to precise positioning of the nucleosomes. Arguments have been advanced in favor of both sequence-determined phasing (9, 34) and factor-determined (32) or statistical (16) phasing. Data currently available for the MMTV LTR do not permit a critical determination of the mechanism of phasing for this sequence.

The conclusions of greatest interest in the work presented here relate to the organization of polynucleosomal arrays and their influence on DNA-protein interactions. Transcription factor NF1 apparently cannot bind to its recognition site when that site is accurately positioned on nucleosome B, while GR can recognize one or more of its multiple binding sites. These observations provide a straightforward explanation for the previously described exclusion of NF1 from MMTV chromatin (10). Although the LTR −75 binding site has one of the highest affinities for NF1, and the factor is present in unmodified form in nuclei from uninduced cells, the protein only binds to its site in vivo in the presence of receptor.

Two observations argue that exclusion of NF1 from the A-B disome is a unique property of the NF1-nucleosome interaction, not a general inhibition of binding by histones. First, the T7(440-525) receptor fragment can bind to the disome, with essentially quantitative conversion of the structure to a tertiary complex. Secondly, *BclI* (whose recognition site is adjacent to the NF1 site in the A-B linker region) is able to cleave the reconstituted disome. DNA sequences quite close to the NF1 site are therefore accessible.

Perlmann and Wrange (28) originally described the in vitro interaction of GR with a reconstituted B-region nucleosome. Pina et al. (29) subsequently confirmed the receptor interaction and also reported, as in our finding, that the NF1 is excluded. With the mononucleosome utilized by Perlmann and Wrange, the octamer core was positioned identically to the B-region core in our experiments. For the monosome reconstituted by Pina et al., however, the core is apparently located 30 nucleotides downstream. This position is not compatible with the nucleosome B position in the in vivo array and would place the NF1 site internal to the B core

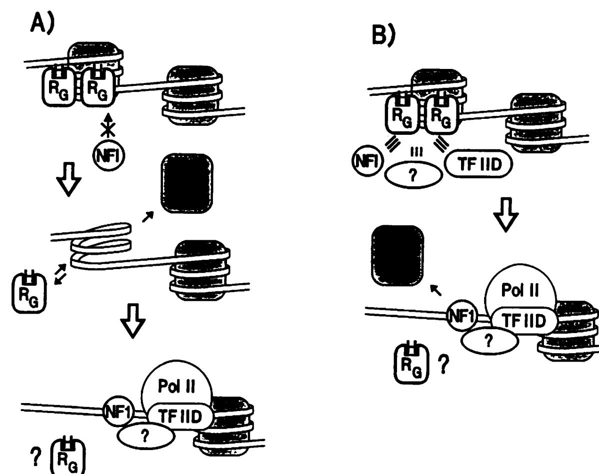

FIG. 6. The A-B nucleosome region during hormone induction. Two possible models are presented to described the structural alterations and factor binding events that occur during glucocorticoid activation at the wild-type MMTV A-B nucleosome domain. H, hormone; R_G, glucocorticoid receptor; Pol II, polymerase II.

region. Since similar factor access results are obtained in each case, the interesting question arises as to whether NF1 exclusion and receptor access result from specific properties of the respective proteins, rather than the rotational position of their binding sites on the octamer core surface. This question is now amenable to direct experimental testing.

Our present understanding of MMTV promoter chromatin is modeled in Fig. 6. The wild-type MMTV promoter is activated through an NF1-TFIID initiation complex composed of TFIID, NF1, and potentially other undescribed factors. Workman and Roeder (35) have argued that preassembly of nucleosomes can inhibit the binding of TFIID, but for the MMTV LTR the TFIID binding site is located in wild-type MMTV chromatin on the left side of nucleosome A, partly in the A-B linker region. TFIID could presumably bind alone if it possessed a higher intrinsic affinity for the promoter. NF1, however, is excluded by the presence of nucleosome B as a result of the specific organization of its binding site in the nucleosome B structure. Although the receptor binding site is also located on nucleosome B, it must be organized in such a way that it remains accessible to the receptor protein. GR can therefore bind and initiate a process that leads to the ultimate displacement of nucleosome B. The nucleosome apparently must be displaced to permit NF1 binding and subsequent assembly of the initiation complex. Simple binding of receptor in vitro clearly does not displace the octamer core (Fig. 5B). Binding of receptor to mononucleosomes does result in subtle alterations in nuclease cleavage patterns of the receptor-mononucleosome complex that could have implications for displacement in vivo (28, 29).

Two general mechanisms for nucleosome displacement are compatible with current data. Displacement could be a separate and distinct event, initiated uniquely by receptor and other transcription factors with similar capabilities (Fig. 6A). Alternatively, the process could be driven by protein-protein contacts (30) between receptor and some member of the initiation complex. This interaction would in turn alter the equilibrium of the macromolecular assembly to favor nucleosome displacement (Fig. 6B). What is clear from the

available evidence, however, is that the nucleoprotein molecule in the living cell is not a neutral template. The promoter is organized in such a way that basal, constitutive transcription is suppressed through a mechanism that results in the exclusion of factors from chromatin.

The present discussion is pertinent only in consideration of genes in a stable nucleoprotein structure. We do not know to what extent transiently introduced DNA is organized in nucleoprotein structures reflective of stable chromatin, nor can we determine what fraction of this DNA actually serves as a template. Results from these systems may selectively emphasize the role of protein-protein contacts, although replication of the DNA template has been shown to be important for some responses even in transient expression analysis (6, 24). In this regard, we have recently demonstrated that hyperacetylation of histones causes the MMTV promoter to become refractory to hormone stimulation and nucleosome displacement (7). If the MMTV promoter is introduced transiently into cells, however, no effect is observed; the transient promoter is fully inducible. These results further emphasize the complexity of the interaction between soluble factors and the nucleoprotein template.

The central conclusion from our experiments is that transcription factor access to the MMTV promoter is modulated by accurately positioned nucleosomes in vitro. This result is consistent with our previous experiments which suggest that the GR induces nucleosome displacement in vivo, leading to the formation of a preinitiation complex and the onset of transcription. The point of greatest interest now is to establish the mechanism through which displacement occurs. This issue can best be addressed by reconstitution of nucleoprotein molecules in vitro with accurately positioned nucleosomes and the assembly of a cell-free nucleosome displacement system.

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