Nucleosomes Reconstituted *in Vitro* on Mouse Mammary Tumor Virus B Region DNA Occupy Multiple Translational and Rotational Frames

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ABSTRACT: The mouse mammary tumor virus acquires a highly reproducible chromatin structure when integrated into cellular DNA. Previous studies have suggested that the LTR is arranged as a series of six phased nucleosomes, that occupy specific positions on the LTR. On the basis of nucleosome reconstitution studies using DNA from the B region of the LTR, it has been argued that this sequence directs a uniquely positioned nucleosome. Here we demonstrate *in vitro* that reconstituted B region nucleosomes adopt at least five distinct translational positions in two rotational frames on a 206 bp fragment of DNA. We have resolved an initial reconstitute into its component species using nondenaturing gel electrophoresis, and precisely mapped the positions of each species using a hydroxyl radical footprinting assay. To confirm the nucleosome positions determined with the hydroxyl radical assay, nucleosome boundaries were mapped using exonuclease III. Comparison of the results from the hydroxyl radical footprinting and exonuclease III assays revealed a symmetrical pattern of overdigestion by exonuclease III which made unequivocal determination of nucleosome boundaries dubious. We conclude that the general use of exonuclease III to map the positions of nucleosomes may lead to incorrect assignment of position, and that assignment of position through the determination of the nucleosome pseudo-dyad from hydroxyl radical footprinting data represents a superior method of analysis.

DNA in eukaryotic cells is complexed with histone proteins to form chromatin. Chromatin plays an essential role in the packaging and condensation of DNA within the nucleus, and has been shown to regulate the transcriptional activity of specific genes [for reviews, see Grunstein (1990) and Felsenfeld (1992)]. The fundamental unit of chromatin structure is the nucleosome core, and contains 146 base pairs (bp)[1] of DNA wrapped 1.75 turns around a histone octamer containing a pair of each of the 4 histone core proteins (Richmond et al., 1984). Because of the helical nature of the DNA molecule, any specific sequence of nucleosome DNA is related to the octamer core by two properties. The translational phase of a nucleosome describes the 146 bp of linear DNA sequence that is wrapped on the octamer core, and reflects the specific histone environment that a particular segment of that sequence interacts with. The rotational phase of a nucleosome describes the relationship of the DNA helix to the octamer core and, hence, the extent to which a specified sequence is oriented toward or away from the protein core. Because nucleosomal DNA exhibits an average helical periodicity of approximately 10 bp (Drew & Travers, 1985; Hayes et al., 1990; Satchwell et al., 1986), all nucleosomes that differ in their translational phase by a multiple of 10 bp share a common rotational phase.

The mouse mammary tumor virus (MMTV) LTR has been well characterized as a steroid-inducible transcription unit (Hager, 1988; Beato, 1989; Hager & Archer, 1991; Gunzburg & Salmons, 1992), and more recently has provided the field of chromatin with an important model for the regulation of transcription by histones [see Hager et al. (1993) and references cited therein]. Current understanding of the nucleoprotein structure of the MMTV LTR is based on data obtained by low-resolution mapping of nucleosome positions *in vivo* (Richard-Foy & Hager, 1987; Bresnick et al., 1991; Truss et al., 1993), and by reconstitution of small fragments of the LTR with histones *in vitro* (Perlmann & Wrange, 1988; Pina et al., 1990a,c; Archer et al., 1991). The resulting model of LTR chromatin structure incorporates the LTR into an array of six phased nucleosomes (Richard-Foy & Hager, 1987; Perlmann & Wrange, 1988; Pina et al., 1990b). Although this structure has been frequently discussed in terms of uniquely positioned cores, we have recently shown that the positioned micrococcal footprints result not from the occupancy of a unique translational frame, but rather from the biased occupancy of a family of frames for each core (Fragoso et al., 1995). In this context, the B nucleosome region contains four hormone response elements (HREs) and a cognate binding site for the NF-1 transcription factor. The nucleoprotein structure in this region of the LTR is observed to undergo a structural transition that increases the accessibility of DNase I (Richard-Foy & Hager, 1987; Sistare et al., 1987; Bresnick et al., 1990), restriction endonucleases (Bresnick et al., 1991; Archer et al., 1991, 1992, 1994), and MPE-Fe(II) (Richard-Foy & Hager, 1987), in response to treatment with dexamethasone.

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[1] Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; bp, base pair(s); DNase I, deoxyribonuclease I; ExoIII, exonuclease III; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HRE, hormone response element; HR, hydroxyl radical; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; MNase, micrococcal nuclease; MPE-Fe(II), methidiumpropyl-EDTA-Fe(II); NF-1, nuclear factor 1; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride.

Data from several laboratories regarding the *in vitro* reconstitution of nucleosomes containing B region DNA have suggested that a reconstituted nucleosome adopts a single position on the DNA (Perlmann & Wrange, 1988; Pina et al., 1990b; Archer et al., 1991). While the nucleosomes obtained in these studies occupied a common rotational frame, the inferred translational position of the nucleosome on the DNA varied by 30 bp. It was also shown that glucocorticoid receptor could still recognize at least a subset of the nucleosome HREs. By contrast, the higher affinity transcription factor NF-1 failed to interact with its cognate site when reconstituted into a mononucleosome (Pina et al., 1990b), or dinucleosome (Archer et al., 1991). Either an intrinsic property of NF-1 or the specific orientation of the transcription factor binding site with respect to the face of the histone octamer regulates the factor's ability to interact with nucleosomal DNA.

The majority of the assays used to delineate the positions of nucleosomes on the MMTV LTR are based on the altered activity of nucleases on nucleosomal substrates as compared to naked or linker DNA. The reagents DNase I, micrococcal nuclease (MNase), and exonuclease III (Exo III) have classically been used to determine the translational and rotational phase of nucleosomes *in vitro* and *in vivo*. However, one disadvantage to the use of these enzymes is the inherent sequence-specific cleavage preferences that they exhibit (Horz & Altenburger, 1981; Lomonossoff et al., 1981). The sequence-specific cleavage effects are superimposed on the true cleavage pattern obtained from the nucleoprotein structure. The resulting composite pattern makes precise assignments of rotational and, in particular, translational frames often difficult to determine.

In contrast to the sequence-specific cleavage tendencies of nucleases, DNA strand cleavage by the inorganic hydroxyl radical (HR) moiety is sequence-independent and thus yields data that more accurately reflect the nucleoprotein structure of the substrate (Dixon et al., 1991). A second advantage in the use of the HR reagent is the ability to obtain both the rotational and the translational phase of a nucleosome simultaneously. The rotational phase is determined by orienting the cleavage maxima, which occur in the minor groove, away from the octamer surface, while the translational phase is derived from the determination of the nucleosome pseudo-dyad axis as revealed by the characteristic change in the pitch of the DNA helix as it traverses this region (Hayes et al., 1990, 1991b).

We present here an examination of the behavior of reconstituted mononucleosomes on a fragment of DNA encompassing the entire nucleosome B region. We have used low ionic strength nondenaturing gels to separate individual reconstitutes on the basis of mobility together with hydroxyl radical footprinting to characterize the rotational and translational frames adopted by the reconstituted mononucleosomes. We show that in contrast to the previously described simple behavior of reconstituted B region nucleosomes, at least five nucleosome positions are shown to occur in two different rotational phases.

EXPERIMENTAL PROCEDURES

Cell Culture. The 904.13 cell line was used as the source of histones for these studies. This cell line was derived from C127 mouse mammary epithelial cells and contains a tandem array of approximately 200 integrated copies of the MMTV LTR driving the Ha-ras oncogene (Bresnick et al., 1992). The cells were grown in Dulbecco's DMEM containing 10% fetal calf serum and penicillin, streptomycin, and gentamicin antibiotics. Cells were grown to confluence prior to being harvested for the preparation of nucleosome cores.

Preparation of Histone Octamers. Histone octamers were prepared using hydroxylapatite column chromatography as described by van Holt et al. (1989). Nuclei were prepared and suspended in 50 mM Tris-HCl (pH 7.4), 25 mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; ICN Biomedicals, Inc., Costa Mesa, CA] at a concentration of 5 mg/mL (100 A_{260} units/mL) and digested with 40 units/mL micrococcal nuclease (Worthington) for 10 min at 37 °C. The digestion was stopped by the addition of EDTA to 5 mM. The nuclei were then pelleted and resuspended in the original volume of 10 mM Tris-HCl (7.4), 0.25 mM EDTA, and 1 mM AEBSF. The nuclei were disrupted, and the soluble chromatin was extracted using 20 strokes of a Dounce homogenizer fitted with the B pestle. The entire homogenate was then dialyzed overnight at 4 °C against the same buffer containing 0.2 mM PMSF in place of the AEBSF.

The dialysate was centrifuged at 10000*g*, and the supernatant containing the soluble chromatin was loaded onto a hydroxylapatite column equilibrated with 10 mM Na_2HPO_4 (pH 7.4) with 0.2 mM PMSF. After addition of 1 column volume of equilibration buffer to remove nonbound proteins, histone HI and the core histone proteins were eluted with equilibration buffer containing 3 M NaCl. Under these conditions, the elution of HI was well resolved from the peak containing the core histones. The peak containing the core histones was simultaneously concentrated and dialyzed against 10 mM Na_2HPO_4 (pH 7.4), 2 M NaCl, and 0.2 mM PMSF using a Micro-Pro Dicon apparatus fitted with 25 000 dalton cutoff membranes (Spectrum, Houston, TX).

The concentrated dialysate was loaded on a Sephadex G-100 column equilibrated in the above dialysis buffer. All of the detected protein eluted in the octamer peak near the void volume of the column. This material was again concentrated and dialyzed against 10 mM Na_2HPO_4 (pH 7.4), 2 M NaCl, and 0.2 mM PMSF. Core histones were diluted with an equal volume of glycerol and stored at −20 °C at a concentration of 1.2 mg/mL.

Preparation of DNA Fragments. The DNA fragment termed B206 was obtained from a double-stranded DNA PCR product containing MMTV sequences from −240 to −46 (coding strand primer: 5′-TGAATAGCCTTTATTG-GCCCAACC-3′; noncoding strand primer: 5′-AAGCATT-TACATAAGATTTGGATAAATTCC-3′). This PCR product was cloned into the T-tailed cloning vector T7Blue (Novagen, Madison, WI). Typically, 500−1000 μg of CsCl-purified plasmid (pSR151) was digested with a 2-fold excess of *Bam*HI and *Nde*I. The reaction was terminated with the addition of 20 mM EDTA, and the entire sample was loaded on 1.5 × 75 cm Econo-Column (Bio-Rad) containing Bio-Gel-A-1.5 m gel filtration media (Bio-Rad) equilibrated with 10 mM HEPES (pH 7.4), 0.25 mM EDTA (H4E) (Hansen & Rickett, 1989). The eluted fragment peak was concentrated using a MacroSep 30K (Filtron Technology Corp., Northborough, MA) and reapplied to the column. The eluted fragment was concentrated as before, quantitated spectrophotometrically, and stored at −20 °C until use.

End-labeled B206 fragment was obtained by digesting 50 μg of plasmid with either *Bam*HI or *Nde*I in the presence of artic shrimp phosphatase (Amersham, Arlington Heights, IL). After the initial digestion, the phosphatase was inactivated following heat treatment at 75 °C for 15 min. The DNA was then digested with the remaining restriction enzyme and then extracted with phenol/chloroform and precipitated. The precipitated DNA was end-labeled according to standard procedures using 100 μCi of [α-^{32}P]ATP (6000 Ci/mmol, Amersham). Labeled fragment was then separated from the vector in a nondenaturing polyacrylamide gel, visualized by wet gel autoradiography, and eluted.

Salt Dialysis Reconstitution. Reconstitution of mononucleosomes was performed essentially as described by Stein (1989). All reconstitutions contained a total of 10 μg of B206 (or B206-S) fragment and 9 μg of purified histone octamers in a total volume of 100 μL of 10 mM HEPES (pH 7.4), 0.25 mM EDTA (H4E buffer) containing 2 M NaCl. The mixture was dialyzed against H4E containing 800 mM NaCl and 1 mM 2-mercaptoethanol for 90 min at room temperature, followed by dialysis against H4E containing 150 mM NaCl for 90 min at room temperature, and finally dialyzed against H4E buffer, without salt, overnight at 4 °C.

Nondenaturing Gel Electrophoresis. Reconstitutes were resolved on 6% polyacrylamide gels [acrylamide:bis(acrylamide) ratio 37.5:1, National Diagnostics, Atlanta, GA] containing 0.5 × TBE. Generally, 5 μL of sample was loaded per lane followed by electrophoresis at 20 V/cm at 4 °C.

Hydroxyl Radical Footprinting. Footprinting of reconstituted nucleosomes with hydroxyl radical followed the general protocol of Dixon et al. (1991). The Fe(EDTA) reagent was prepared immediately prior to use by mixing equal volumes of 600 μM ferrous ammonium sulfate 6-hydrate and 1200 μM EDTA. For a standard assay, 1 μL aliquots of Fe(EDTA) reagent, sodium ascorbate (10 mM), and hydrogen peroxide (0.3%), and 2 μL of water were applied to the inside wall of a microfuge tube containing 5 μL (500 ng) of reconstitute. The reaction was initiated by combining all of the reagents and continued for 3 min at room temperature. The reaction was then quenched with the addition of 0.1 volume of 100 mM thiourea in 200 mM EDTA. For subsequent nondenaturing gel electrophoresis of the digested samples, 2 μL of glycerol loading dye containing bromophenol blue was added, and the samples were electrophoresed as described above. Gel slices containing the individual reconstitutes were visualized by wet gel autoradiography and excised, minced, and eluted in a buffer containing 100 mM Tris−acetate (pH 7.5), 500 mM NaCl, 5 mM EDTA, and 100 μg/mL proteinase K (Sigma) in order to digest the core histones. The eluted DNA was precipitated by the addition of 2 volumes of ethanol, dissolved in formamide sequencing dye, and loaded on an 8% sequencing gel.

Histone Stoichiometry of the Reconstitutes. For histone composition analysis, 25 μL of reconstitute was resolved by nondenaturing gel electrophoresis as described above. The gel was stained with ethidium bromide, and gel slices containing the three reconstituted species as well as free DNA were excised and equilibrated in sample buffer [60 mM Tris-HCl (pH 6.8), 50% glycerol, 2% SDS, and 0.1% bromophenol blue] for 30 min at room temperature. The equilibrated gel slices were loaded directly into the wells of a discontinuous SDS−polyacrylamide slab gel [stacker: 7.5% acrylamide (acrylamide:bis ratio 29.2:0.8); separating gel: 20% acrylamide (acrylamide:bis ratio 19:1)] and overlayed with additional sample buffer. Purified core histones and total nuclear histones were also loaded. After electrophoresis at 20 V/cm, the gel was fixed in 50% methanol (v/v) containing 10% glacial acetic acid (v/v) and stained with silver by the method of Wray (Wray et al., 1981).

Exonuclease III Assay. Typically, 5 μL of reconstitute was separated in a nondenaturing gel and visualized by wet gel autoradiography. Gel slices containing the individual reconstitutes were equilibrated in 10 mM Tris-HCl (8.0), 3 mM magnesium chloride, and 5 mM 2-mercaptoethanol for 15 min at room temperature. Exonuclease III (New England Biolabs) was added at the desired concentration, and the sample was digested for 30 min at room temperature. The reaction was terminated by the addition of EDTA to 50 mM. The gel slice was then removed from the tube, minced, and eluted under the conditions used for the gel-separated footprinted nucleosomes above. The eluted DNA was precipitated, dissolved in formamide sequencing dye, and loaded on an 8% sequencing gel.

Data Acquisition and Analysis. All radioactive images were obtained using a Molecular Dynamics Phosphorlmager and analyzed with ImageQuant software.

RESULTS

The intent of the current work was to reconstitute the B region of the MMTV genome into a nucleosome using purified histone octamers and a fragment of DNA that was sufficiently large to allow for a variety of possible nucleosome positions instead of requiring the reconstituted nucleosome to fit onto a smaller fragment of DNA chosen a priori. Smaller, core-sized fragments could then be designed based on the derived nucleosomal positions on the larger fragment. Thus, the B206 DNA fragment containing 206 bp of double-stranded DNA encompassing MMTV sequences from −240 to −46 (195 bp of MMTV LTR sequence, Figure 1A) was used in the following studies. Purified octamer cores were reconstituted on the DNA fragment using gradient salt dialysis, and HR footprinting assays were performed in order to characterize the nucleoprotein structure of the reconstitute. The results of the initial footprinting analysis are shown in Figure 1B. The clear modulation of cleavage maxima occurring with a 10 bp periodicity is characteristic of DNA constrained to a nucleosome core (Hayes et al., 1990; Lutter, 1978). Hydroxyl radical cleavage of the DNA is suppressed when the minor groove is oriented toward the surface of the octamer core, while maximal cleavage is obtained when the minor groove is facing toward the solvent. However, we were surprised to find that the nucleosomal cleavage pattern extended over the entire length of the reconstituted DNA fragment. Because a single mononucleosome is expected to associate with 146 bp of DNA, this extended pattern suggested the occurrence of multiple nucleosome species in the reconstituted material, despite the inability of a single 206 bp fragment to accommodate more than one complete octamer nucleosome.

To better understand the nature of the reconstituted material, the sample was subjected to low ionic strength gel electrophoresis (Linxweiler & Horz, 1984). All reconstitutions were resolved into an identical pattern of three

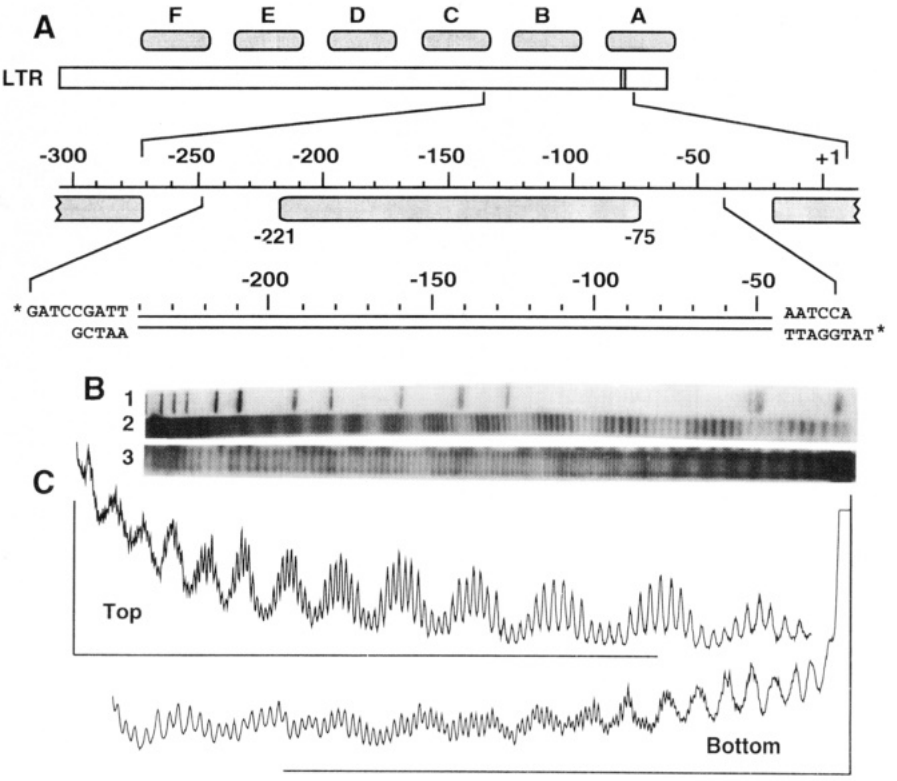

FIGURE 1: Hydroxyl radical footprint of a B206 reconstitute. (A) The B206 DNA fragment is shown in the context of the MMTV B nucleosome family. Base positions are relative to the transcription start site at +1. This fragment contains MMTV LTR sequences from −240 to −46 (shown as double line) and the vector sequences denoted. Due to the difference in the length of the restriction site overhang, the top strand (*Bam*HI) is 210 bp and the bottom strand (*Ndel*) is 208 bp; there is 206 bp of double-stranded DNA. The internal *Sstl* site used in the preparation of B206-S is shown. (B) Hydroxyl radical footprint of the total B206 reconstitute. Lane 1, *Msp*I-digested pBR322 markers; lane 2, hydroxyl radical footprint of B206 top strand; lane 3, hydroxyl radical footprint of B206 bottom strand. (C) Densitometric analysis of the top strand and bottom strand HR cleavage products.

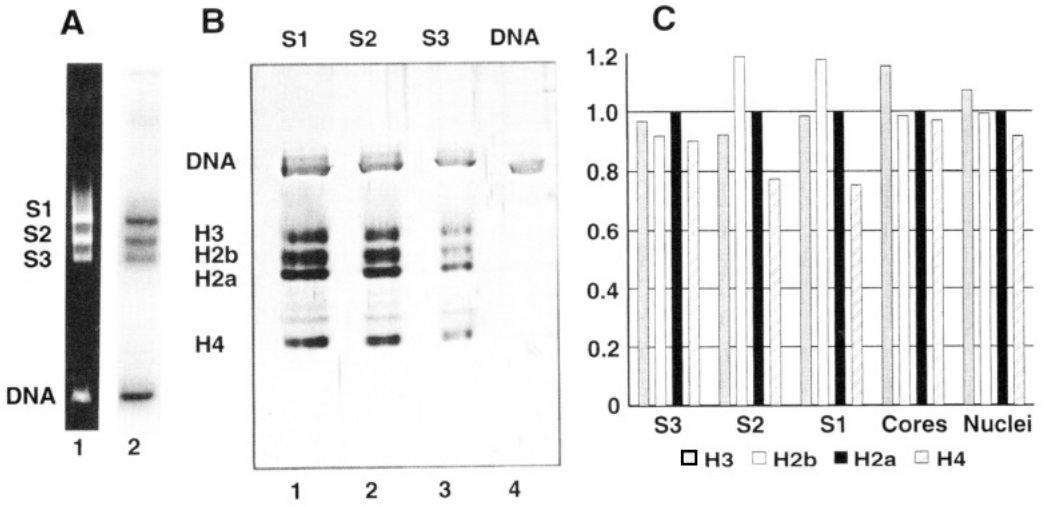

FIGURE 2: Analysis of the individual B206 reconstitute species. (A) Nondenaturing gel electrophoresis of the reconstitute after visualization with ethidium bromide staining (lane 1) or ^{32}P exposure (lane 2). Species nomenclature is indicated on the left. (B) Histone composition of the individual reconstituted species. Proteins associated with each of the resolved reconstituted species were separated by discontinuous gel electrophoresis and stained with silver. (C) Quantitation of the histone stoichiometry. A densitometric scan of the gel in panel B was used to quantitate the relative amounts of the individual histones contained in each species. The relative abundance of each histone protein within a species is expressed as the ratio of that protein to histone H2a, and is compared to values obtained from nuclei and the core histone preparation used in the reconstitution (not shown).

reconstituted complexes and free DNA fragment (Figure 2A). While the relative ratios of reconstituted complexes S1, S2, and S3 (2:1.5:1) were highly consistent between different reconstitutions, the amount of free fragment would vary to a small extent, presumably due to variations in the exact histone:DNA ratios during the reconstitution (data not shown). The histone composition of each of the reconstituted species were determined by resolving the protein component of gel slices containing the separated reconstitutes by discontinuous SDS−polyacrylamide gel electrophoresis (Figure 2B). Each of the reconstituted species contained all four core histones in a stoichiometric ratio that closely reflected that of intact nuclei, and the ratio of histone to DNA appeared to be the same for each of the reconstitutes (Figure 2C). We

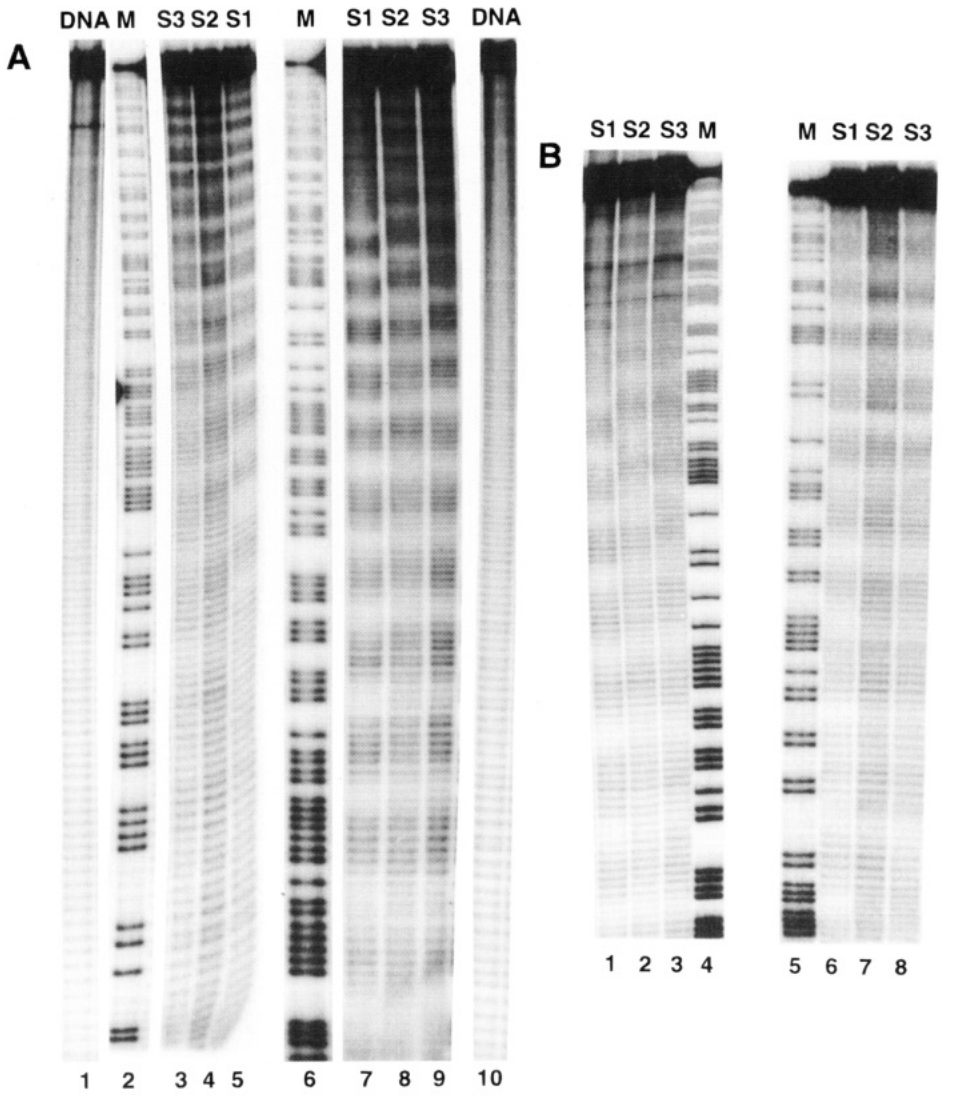

FIGURE 3: Hydroxyl radical footprints of the individual species of the B206 reconstitute. The total reconstitute was subjected to cleavage by hydroxyl radical and subsequently resolved into the individual components on a nondenaturing gel. The DNA cleavage products were then extracted and resolved on an 8% sequencing gel. (A) Short electrophoresis run of cleavage products. Lanes 1−5, top strand cleavage products from naked DNA (lane 1), the Maxam−Gilbert purine-specific sequencing reaction (lane 2), and the indicated reconstituted species (lanes 3−5). Lanes 6−10, bottom strand cleavage products from the Maxam−Gilbert purine-specific sequencing reaction (lane 6), the indicated reconstituted species (lanes 7−9), and naked DNA (lane 10). (B) Longer electrophoresis run of cleavage products. Lanes 1−4, top strand cleavage products from the indicated reconstituted species (lanes 1−3) or the Maxam−Gilbert purine-specific sequencing reaction (lane 4). Lanes 5−8, bottom strand cleavage products from the indicated species (lanes 6−8) or the Maxam−Gilbert purine-specific sequencing reactions for the bottom strand (lane 5).

have previously demonstrated that authentic mononucleosomes from a partial MNase digestion of intact nuclei displayed a mobility in a nondenaturing gel that closely approximated that of the reconstituted species, and that isokinetic sucrose gradient analysis of the total reconstitute showed the reconstituted material sedimenting at 11 S (data not shown). Thus, reconstitution of mononucleosomes with purified histones on the 206 bp B region DNA fragment resulted in the formation of three different reconstituted mononucleosomal species that could be resolved on the basis of mobility in a nondenaturing gel. These results suggested that the HR digestion pattern shown in Figure 1B,C reflects the sum of patterns from individual components of the reconstitute, thereby explaining the modulation of HR cleavage over the entire length of the fragment.

We next sought to evaluate the basis for the mobility differences of the reconstituted mononuclesomes observed in Figure 2A. Because the cleavage reaction was performed under conditions that generated an average of less than one cleavage event per DNA strand [greater than 80% of the probe remains undigested in Figure 1B,C (Brenowitz et al., 1986)], we reasoned that prior digestion of the total reconstitute with HR would not subsequently affect the mobility or character of the individual reconstituted species during gel electrophoresis. After resolving the digested reconstitutes in a nondenaturing gel, the nucleosomal DNA was deproteinized and extracted from the gel slices for analysis (Figure 3). The digestion pattern of the naked DNA fragment was essentially featureless, indicating a lack of intrinsic DNA structure as assayed by this reagent. In contrast, each of the reconstituted species demonstrated a clear pattern of cleavage products that is characteristic of nucleosomal DNA. Inspection of the data from this analysis (Figure 3) reveals the following points. First, the periodicities of the three reconstituted species are observed to be in phase with each other at the 3′ end of the DNA fragment (lower region of the bottom strand gel, Figure 3B; upper region of the top strand gel, Figure 3A). However, species

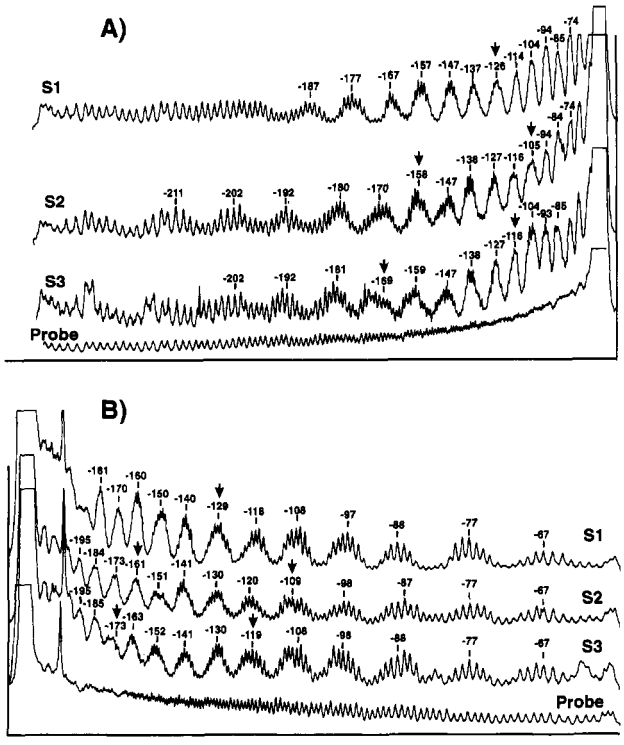

FIGURE 4: Densitometric analysis of the cleavage products shown in Figure 3. Peak assignments are a composite of those obtained from the short and long electrophoresis runs of the hydroxyl radical cleavage products. The nucleosomal pseudo-dyad(s) is(are) indicated by a boldface arrow. (A) Top strand cleavage pattern. (B) Bottom strand cleavage pattern.

S2 and S3 are out of phase with S1 in the upstream region of the fragment. Furthermore, while the S1 species contains a region at the upstream end of the DNA fragment with the appearance of naked DNA (at the bottom of the top strand gel), species S2 and S3 exhibit a continuous, oscillating cleavage pattern over the fragment's entire length, analogous to that obtained with the unfractionated reconstitute (Figure 1B,C). We also note that while HR cleavage of species S1 is very strongly suppressed when the minor groove is facing toward the octamer surface, species S2 and S3 contain comparatively stronger band intensities in the analogous positions.

The scans of the HR cleavage patterns for the individual reconstituted species are shown in Figure 4 along with the peak assignments for maximal cleavage, i.e., minor groove facing away from the octamer core. HR digestion of species S1 yielded data that are consistent with the occurrence of a single nucleosome located toward the 3′ end of the DNA fragment. On the top strand of species S1 (Figure 4A), a clear 10 bp periodicity is apparent from position −74 to −114; between −115 and −137, there is an ncreased periodicity of 11−12 bp over two turns of the DNA helix. A 10 bp periodicity is then reestablished starting at −147 until the pattern becomes isotropic after −187. The positions of maximal cleavage on the bottom strand are completely complementary but are offset in the 3′ direction (on the bottom strand) approximately 3 bp relative to the top strand positions, indicative of minor groove-specific cleavage chemistry (Lutter, 1977). The nucleosomal pseudo-dyad position, or center of the 146 bp core DNA sequence, was determined from the positions of increased helical periodicity (Hayes et al., 1990, 1991b), which corresponds to position −126 on the top strand and −129 on the bottom strand. The center of this transition was taken as −127 and represents the S1 pseudo-dyad. Given the center of the nucleosome core at −127, the core boundaries can then be defined as −200/−55. The derived upstream boundary coincides closely with the end of the oscillating HR cleavage pattern at −187. Although position −187 is the last cleavage maximum that could be assigned, a clear suppression of HR cleavage can be observed at position −193, strongly suggesting the involvement of another turn of the helix with the octamer core. Micrococcal nuclease mapping of the S1 species indicated boundaries at −199 and −54, completely consistent with those determined from the hydroxyl radical data (data not shown).

The analysis of the S2 and S3 species was performed in a method analogous to that used for S1. Inspection of the plotted cleavage pattern of species S2 and S3 reveals a generally similar pattern to that of S1 (Figure 4A,B). However, S2 and S3 both differ from S1 in several important ways. While all three of the reconstituted species are in the same rotational phase near the 3′ end of the fragment (from −65 to −96 on the top strand), species S2 and S3 are observed to be shifted in the rotational phase relative to S1 in the upstream region of the fragment (this is most easily seen in the top strand plots of Figure 4A, between positions −165 and −190). Species S2 and S3 also differ from S1 in the extension of the HR cleavage pattern well beyond the 5′ end of the S1 pattern, effectively covering the entire fragment. Analysis of species S2 in the 3′ region of the DNA fragment clearly reveals a pseudo-dyad at position −107, which correspondingly positions a nucleosome at −180/−34. Thus, the 3′ end of the nucleosome core extends 6 bp off of the end of the double-stranded region of the DNA fragment, though an additional 2 bp of single-stranded DNA are present on the bottom strand. The occurrence of a nucleosome core boundary beyond the end of a DNA fragment has previously been observed, and probably reflects the presence of specific positioning signals within the target DNA (Nobile et al., 1986; Ramsay et al., 1984; Wolffe & Drew, 1989). While somewhat less distinct, the pseudo-dyad of species S3 was similarly mapped to position −117, which corresponds to a nucleosome positioned between −190/−44. Thus, the derived positions of these three nucleosomes represent a family of rotationally phased particles that occur at 10 bp intervals between positions −200 and −34.

As stated above, the nucleosomal cleavage patterns of species S2 and S3 differ from that of S1 in that they appear to extend over the entire DNA fragment. In both species, a 10 bp periodicity is reestablished upstream of the pseudo-dyad, but in contrast to S1, a second change in periodicity, upstream of the pseudo-dyads identified above, was also observed (Figure 4A). This indicates that S2 and S3 were in turn composed of multiple species. We have mapped the positions of these secondary nucleosomes by their deduced pseudo-dyad positions to positions −160 for S2, and −170 for S3. The corresponding nucleosome positions are then −233/−88 and −243/−98 for S2 and S3, respectively. Because it is not possible for one molecule of the DNA fragment to be occupied simultaneously by the upstream and downstream nucleosomes identified in species S2 or S3, we postulate that the bands identified in the nondenaturing gel as species S2 or S3 each represent two, comigrating,

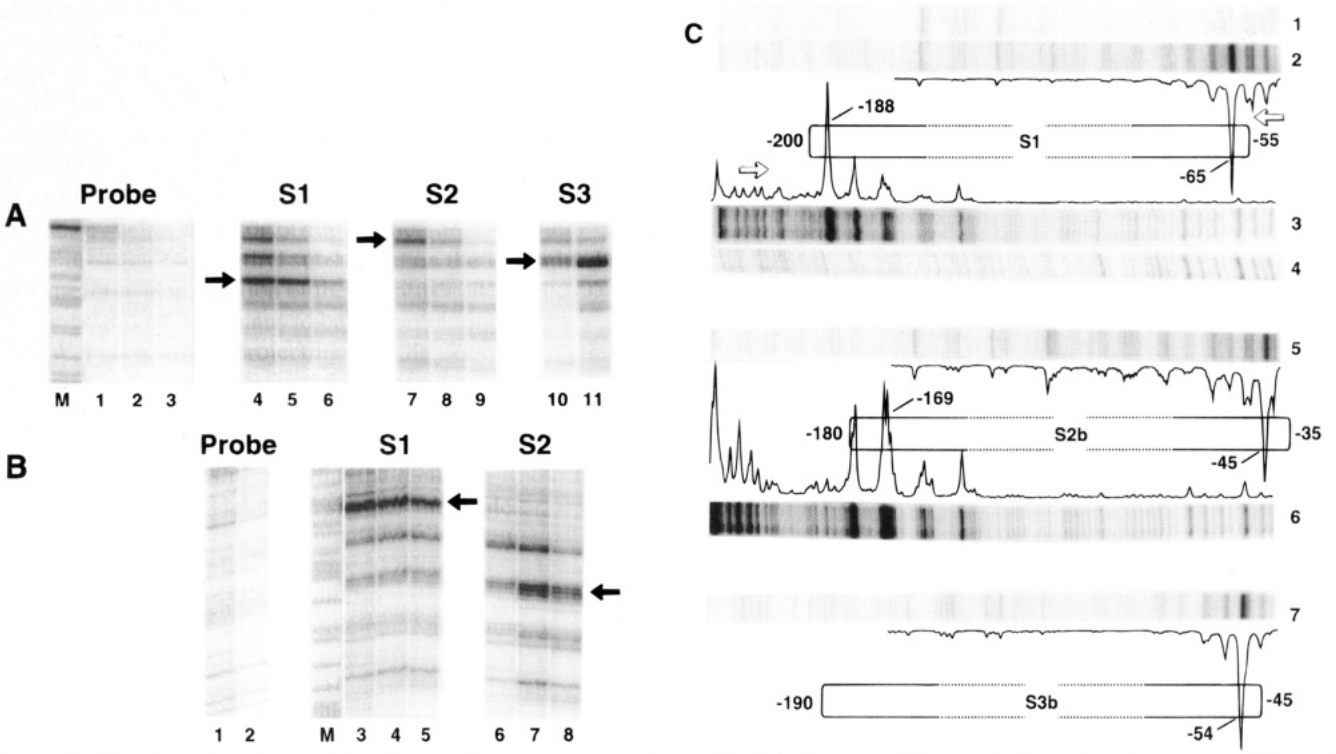

FIGURE 5: Mapping of nucleosome boundary positions using exonuclease III. Gel slices containing the indicated species were equilibrated with ExoIII digestion buffer and digested with ExoIII as described under Experimental Procedures. (A) Top strand cleavage products from naked B-206 DNA (lanes 1−3) or reconstituted species S1 (lanes 4−6), S2 (lanes 7−9), and S3 (lanes 10 and 11) using ExoIII at 10 units/mL (lane 1), 30 units/mL (lanes 2, 4, 7, and 10), 100 units/mL (lanes 3, 5, 8, and 11), or 300 units/mL (lanes 6 and 9). A Maxam−Gilbert purine-specific sequencing reaction was used to identify boundary positions (lane M). The arrows indicate the primary ExoIII stops for S1(−65), S2(−45), and S3(−54). (B) Bottom strand cleavage products from naked B-206 DNA (lanes 1−2) or reconstituted species S1 (lanes 3−5) and S2 (lanes 6−8) using ExoIII at 10 units/mL (lanes 3 and 6), 30 units/mL (lanes 1, 4, and 7), or 100 units/mL (lanes 2, 5, and 8). The arrows indicate the primary ExoIII stops obtained for S1(−188) and S2(−169). Nucleosome-specific stops were not obtained on the bottom strand of species S3. (C) Alignment of ExoIII digestion products with the HR-determined nucleosome position of species S1, S2b, and S3b. The complete set of ExoIII stops are shown above (top strand) and below (bottom strand) each of the indicated nucleosomes, along with a densitometric plot of the data. The digestion products obtained with naked DNA are shown above and below species S1 for reference (1 and 4, 10 and 30 units/mL, respectively). No densitometric plots of these data are shown. Data for species S1 and S3b were from the 100 units/mL samples; data for species S2b were from the 30 units/mL samples. Arrows indicate the direction of ExoIII digestion.

mononucleosome populations. Furthermore, because near the ends of the DNA fragment the nucleosome-specific oscillation of the HR cleavage pattern of one population would be dampened by the naked DNA signal derived from the corresponding nucleosome, the two mononucleosome populations within each gel species must be represented in approximately equivalent amounts. We have termed the upstream and downstream nucleosomes S2a and S2b, and S3a and S3b, respectively. The rotational frame of the upstream nucleosomes, S2a and S3a, is 3 bp upstream of the rotational frame of the three downstream nucleosomes (S1, S2b, and S3b). It is because of this particular relationship between the rotational phases of the upstream and downstream nucleosomes that an apparently unambiguous cleavage pattern could be obtained from the total, unresolved reconstitute. The superimposition of nucleosomal cleavage patterns that differ in rotational phase would ordinarily result in signal interference, and uninterpretable rotational information. However, because a phase transition of approximately 3 bp occurs within a single mononucleosome as the DNA traverses the nucleosomal pseudo-dyad, the cleavage patterns of the overlapping downstream and upstream halves of nucleosome pairs differing in rotational phase by 3 bp would tend to coincide, maintaining rotational information (see Figure 7). The significance of this point is discussed below.

Because of the surprising complexity of the reconstituted population, we also used ExoIII to independently map the boundaries of each of the nucleosome species. ExoIII is a double-stranded DNA-specific 3′-exonuclease that will digest chromatin until the penetration of the enzyme is hindered by the presence of a nucleosome (Chao et al., 1979). On isolated, mononucleosomal substrates, the enzyme will enter the core DNA sequence, pausing at 10 bp intervals (Prunell, 1983; Prunell & Kornberg, 1978; Linxweiler & Horz, 1982). Digestion of the total reconstitute with ExoIII prior to electrophoresis in nondenaturing gels was shown to be detrimental to the subsequent resolution of the individual reconstitutes (data not shown); therefore, the digestion of the reconstitutes was performed on isolated gel slices containing each of the separated species. Digestion of species S1 with varying concentrations of ExoIII resulted in nucleosome-specific stops, or pauses, on the top strand at −45, −54, and −65. Of these, the strongest and most resistant stop was at −65 (Figure 5A). Similarly, digestion of the bottom strand of S1 was stopped at positions −188, −178, and −169 primarily, with the −188 stop being the most prominent. The 10 bp periodicity exhibited in both sets of these bands is further evidence for the presence of nucleosomal DNA substrate in a single rotational phase. However, the strongest ExoIII stops for species S1 (−188

and −65) represent a protected fragment of only 124 bp, a length that is well short of the canonical 146 bp of DNA associated with a nucleosome core particle. Furthermore, these boundaries do not correspond with the nucleosomal boundaries (−200/−55) deduced from the pseudo-dyad position defined with the HR reagent. There is, nevertheless, a striking symmetry in the comparison of the HR- and ExoIII-determined boundaries; i.e., the prominent ExoIII-derived boundaries represent positions that are approximately 10 bp inside of the HR-derived nucleosomal positions.

When the composite species S2 was mapped with ExoIII, strong stops were observed at −45 on the top strand and, most strongly, at −169 on the bottom strand. These boundaries correlate well with the subspecies S2b mapped by HR, but again are symmetrically positioned 10 bp inside of the HR-defined nucleosome position. Note that although the downstream edge of the S2b nucleosome extends beyond the end of the DNA fragment, the penetration of ExoIII into the core results in a stop on the DNA fragment at position −45. Surprisingly, no other nucleosome-specific boundaries were obtained that could be correlated with subspecies S2a. Identical results were obtained (for S1 and S3b, as well as for S2b) when the separated mononucleosomal species were cross-linked in the gel prior to treatment with ExoIII (data not shown).

Analysis of the composite species S3 with ExoIII revealed a strong boundary stop at −54 on the top strand. The HR-derived downstream position of this nucleosome is −44, so that the ExoIII boundary is again 10 bp inside of the HR boundary for nucleosome subspecies S3b. No corresponding boundary was observed for the upstream boundary of nucleosome S3b (data not shown). Additionally, no specific ExoIII stops were observed for the upstream subspecies S3a.

Because species S2a and S3a were not observed in the ExoIII mapping experiments, we sought additional evidence that this alternative rotational frame could be adopted on this DNA sequence. Nucleosomes were reconstituted on a 137 bp fragment (B206-S, −245/−109) that essentially corresponds to the nucleosomal DNA of subspecies S3a. Hydroxyl radical analysis of the resultant reconstitute verified the occurrence of a nucleosome with the identical rotational phase as subspecies S2a and S3a (Figure 6). The establishment of the rotational phasing of subspecies S2a and S3a in this reconstitute provides strong evidence for the presence of those nucleosomal positions in the original reconstitute with the 206 bp B region DNA fragment, and clearly demonstrates the potential for this DNA to adopt more than one rotational phase.

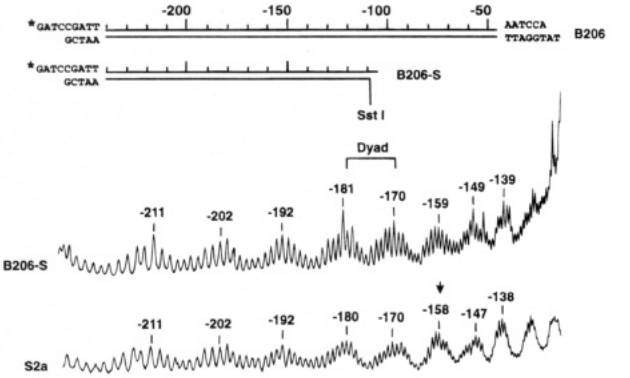

FIGURE 6: Nucleosomes reconstituted on the B206-S DNA fragment are in the same rotational frame as species S2a and S3a. The relationship of the 137 bp B206-S DNA fragment to B206 is shown at the top. Nucleosomes reconstituted with end-labeled B206-S were footprinted with hydroxyl radical under standard conditions. Below are densitometric plots of the separated cleavage products of the B206-S reconstitute and, for comparison, species S2a (from Figure 4A). The pseudo-dyad transition of the B206 reconstitute occurs within the bracketed region. The arrow denotes the pseudo-dyad position of species S2a.

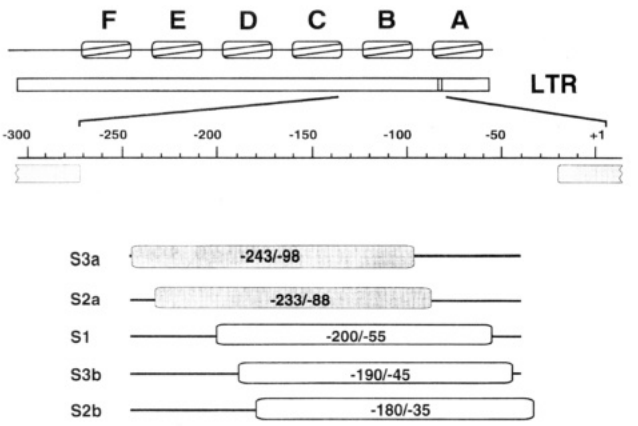

FIGURE 7: Summary of nucleosome heterogeneity *in vitro*. The approximate low-resolution positions of the six MMTV LTR nucleosomes are shown above the LTR at the top of the diagram. An expanded region of the nucleosome B region of the LTR is shown in the middle of the figure above a scale representation of the *in vitro* nucleosome positions determined in this study. The common rotational frame of the two most upstream positions is distinguished from that of the three downstream positions by the shading.

DISCUSSION

We have reconstituted nucleosomes on a 206 bp DNA fragment encompassing MMTV LTR B region DNA using purified histone octamers and have analyzed the resultant nucleosomes using nondenaturing gel electrophoresis, hydroxyl radical footprinting, and limited ExoIII digestion. Our results clearly demonstrate the occurrence of two families of nucleosome positions that differ with respect to rotational phase and contain a total of five members that are translationally positioned in 10 bp intervals (see Figure 7).

The establishment of multiple, precisely positioned nucleosomes during *in vitro* reconstitution has been previously described using a variety of naturally occurring and synthetic DNA sequences (Dong et al., 1990; Linxweiler & Horz, 1984; Zhang & Horz, 1984). In particular, our laboratory has previously postulated the presence of multiply positioned nucleosomes on a synthetic nucleosome B region DNA fragment (Bresnick et al., 1991). It is noteworthy that while a strong tendency for nucleosomes to reconstitute at the end of the target DNA fragment has been observed (Linxweller & Hörz, 1984, 1985), the predominant species observed in the current study (S1) is in the most centrally located position (−200/−55), ruling out end effects as a primary determinant of nucleosome position in our experiments. Furthermore, the positioning of a nucleosome boundary that extends beyond the end of the DNA fragment (S2b) has also been reported (Nobile et al., 1986; Ramsay et al., 1984; Wolffe & Drew, 1989). The presence of this species provides evidence for the existence of positioning signals within the DNA fragment that can overcome the energy loss resulting from lost DNA−protein contacts.

Although previous studies have examined the *in vitro* structure of reconstituted B region nucleosomes using DNA fragments significantly larger than 146 bp, a unique nucleosome position was reported in each of those studies (Perlmann & Wrange, 1988; Pina et al., 1990b). Using a 180 bp fragment, Pina et al. reported a nucleosome at the position of species S3b (−190/−45), the least represented species in the current work (Pina et al., 1990b). In contrast, the nucleosome position reported by Perlmann and Wrange (−219/−76) was not obtained in the current experiments, although this nucleosome exhibits the same rotational phase as species S1, S2b, and S3b (Perlmann & Wrange, 1988). However, it is important to note that in the above study the reconstitution was performed by a different protocol involving the high-salt exchange of histones from stripped chromatin. Moreover, the DNA fragment used for reconstitution contained MMTV DNA sequences only from −200 to −59, and also contained a bacteriophage T7 promoter element at the 5′ end of the fragment. The T7 promoter element has subsequently been shown to direct the rotational and, possibly, the translational positioning of reconstituted nucleosomes (Hayes et al., 1991a). Coincidentally, the spacing of the T7 promoter segment in that DNA fragment resulted in a T7 promoter-directed rotational phase that exactly matched the naturally occurring phase obtained with species S1, S2b, and S3b. The translational position may have also been directed to a unique position by the presence of the T7 promoter element, because the MMTV DNA sequence contained in that fragment (−200/−59) is essentially identical to that occupied by species S1 in the present work (−200/−55), yet no nucleosome occupying the position of S1 was observed.

The apparent discrepancy between our work and previous studies regarding position heterogeneity of reconstituted B region nucleosomes may result from the inability to resolve a heterogeneous reconstitute under certain conditions, and from the particular spatial relationship between the two rotational families defined in this study. In the previous studies, nucleosome reconstitutes either were marginally resolved from free DNA or were separated on nondenaturing gels that contained significant concentrations of glycerol. It has subsequently been demonstrated that electrophoresis of a mixed reconstitute in nondenaturing gels containing glycerol impairs the resolution of the individual species (Pennings et al., 1992). As stated earlier, the two rotational families identified in this work differ in phase by 3 bp. Because of this unique arrangement, digestion of a heterogeneous reconstitute with DNase I or HR would result in overlapping signals from species S2a (or S3a) and S1, S2b, and S3b that would tend to coincide, giving the impression of a single rotational frame. Furthermore, because digestion of isolated mononucleosomes with ExoIII does not typically result in the generation of a unique resistant fragment, it would be difficult to distinguish the digestion pattern of a mixed reconstitute containing nucleosomes separated by 10 bp from that of a uniquely positioned nucleosome. Thus, it is possible that a heterogeneous population of nucleosomes was also obtained, but not identified, in the previous studies.

The predominant rotational phase exhibited by S1, S2b, and S3b has also been observed in a study involving the *in vitro* integration of the murine leukemia virus into reconstituted MMTV Nuc B region nucleosomes (Pryciak & Varmus, 1992), where viral integration occurs preferentially in the exposed major groove of bent DNA (Pryciak et al., 1992; Muller & Varmus, 1994). Both the DNase I cleavage pattern and the sites of integration were consistent with the rotational phase of species S1, S2b, and S3b; translational positioning of the cores was not addressed in that study.

The results of the boundary mapping experiments using ExoIII strongly supported, but did not coincide with, the HR-deduced nucleosome positions for S1, S2b, and S3b. The consistent, strong penetration of ExoIII 10 bp beyond the putative boundary of the mononucleosomes may not be surprising considering the isolated nature of each particle. The free DNA ends may possess additional degrees of freedom that normally do not occur in a nucleosomal array, allowing the enzyme to peel away the outermost DNA from the octamer core. It is clear that a high degree of bias can be introduced into ExoIII (and to a lesser degree DNase I) assays, either through sequence-specific cleavage effects or through manipulation of the digestion conditions. For these reasons, we would argue that it is unreliable to assign nucleosome boundaries based primarily on the ExoIII assay. The assay may be most useful when used to confirm deduced nucleosome positions instead of in the initial determination of position. Note that "confirm" in this context does not necessarily signify identity with previously established boundary positions, but rather a concordance in position based primarily on symmetry considerations as opposed to absolute boundary assignments.

The inability of ExoIII to detect any of the four boundaries associated with species S2a and S3a remains puzzling. HR cleavage was performed in solution, prior to gel electrophoresis, while ExoIII analysis was carried out after gel separation. The most plausible explanation is that the S2a and S3a species are relatively unstable and are disrupted either by the action of ExoIII or during electrophoresis on the nondenaturing gel. Nonetheless, less stable rotational or translational frames detected *in vitro* could potentially become important positions *in vivo* through the action of boundary effects, or the exclusion of core positions found to be more stable *in vitro*.

The glucocorticoid receptor recognizes its cognate binding site through sequence-specific interaction of the protein with two successive major grooves of the DNA helix (Luisi et al., 1991). It is reasonable to expect that the orientation of the glucocorticoid response element with respect to the face of the nucleosome octamer could regulate the ability of the protein to interact with a nucleosomal binding site. Previous work has demonstrated that the affinity of an isolated GRE within the context of a synthetic, artificially positioned nucleosome can be modulated by the translational position of the GRE (Li & Wrange, 1993). In light of the observed heterogeneity of nucleosome positions *in vitro*, determining the effect of the translational and rotational setting of a nucleosome on the ability of GR to interact with its binding site remains of primary interest in elucidating the mechanism of steroid induction of chromatin templates.

In related work, we used micrococcal nuclease in conjunction with formaldehyde cross-linking to provide a high-resolution map of the *in vivo* positions of the A and B nucleosomes (Fragoso et al., 1995). We clearly establish that *in vivo* the A and B nucleosomes each occur in a highly reproducible, nonrandom array of positions. The previously observed low-resolution phasing of nucleosomes on the MMTV LTR results from the preferred occupancy of a subset

of the translational frames identified in that study. Unfortunately, the limitations of the micrococcal nuclease assay preclude the possibility of determining the rotational phase, or exact translational position of any of the identified nucleosome positions. Our demonstration here that following *in vitro* reconstitution a variety of translational and rotational phases are reproducibly occupied on B region DNA further establishes the manifold chromatin structure of this DNA region.

As is the case with MMTV (Richard-Foy & Hager, 1987; Perlmann & Wrange, 1988; Pina et al., 1990b), the 5S gene of a number of organisms was believed to phase nucleosomes in a translationally specific pattern (Gottesfeld, 1987; Roberson et al., 1989; Simpson & Stafford, 1983; Thoma & Simpson, 1985; Rhodes, 1985). When the nucleoprotein structure of the conserved 5S gene of *Saccharomyces cerevisiae* was recently examined both *in vitro* and *in vivo*, nucleosomes were also observed to occupy a variety of translational frames (Buttinelli et al., 1993). An interconversion of nucleosome positions has been established *in vitro* with sea urchin 5S rDNA (Pennings et al., 1991), and bulk mononucleosomes (Meersseman et al., 1992). The apparently low energy barrier restraining nucleosomes to a specific translational position probably explains the occurrence of multiply positioned nucleosomes in these systems as well as in the present work.

Rotational phasing of nucleosomes has been determined on the *Xenopus borealis* 5S rDNA gene (Rhodes, 1985; Hayes et al., 1990). Interestingly, naked 5S rDNA exhibited an HR cleavage pattern that was remarkably similar, albeit attenuated, to the pattern obtained from the reconstituted DNA, strongly suggesting that the naked DNA was "prebent" into the structure manifested in the nucleosome (Hayes et al., 1990, 1991b). The complete absence of such structure in the naked B206 fragment may correlate with the ability of this DNA to accommodate a nucleosome in more than one rotational setting. Despite the lack of structure in the naked DNA fragment, theoretical calculations and experimental evidence have suggested that nucleosome B region DNA has a intrinsic bendability that closely approximates the DNA structure acquired in species S1, S2b, and S3b (Pina et al., 1990a,d). The intrinsic bendability of a DNA fragment is not necessarily reflected in the structure of a reconstituted nucleosome, however. Using HR to probe DNA structure, Hayes et al. showed that DNA molecules with very different structures in solution, and on calcium phosphate crystals, are nevertheless incorporated into nucleosomes of identical structure (Hayes et al., 1991a). Similarly, the identification of a preferred rotational orientation of naked nucleosome B region DNA does not preclude the existence of other orientations following nucleosomal reconstitution.

Many of the central questions regarding the role of chromatin in the regulation of the MMTV LTR remain unchanged. What aspects of the chromatin template control differential access of transcription factors to DNA? What is the status of H1 and core histones after the steroid-induced structural transition? It is likely that the hormone-induced transition is dynamically complex, involving more than one structural intermediate. Our demonstration of multiply positioned nucleosomes *in vitro* and *in vivo* (Fragoso et al., 1995) should cause the reevaluation of these questions in the context of nucleosome populations. In particular, special emphasis will have to be given to the identification of subpopulations that are able to participate in the biological event under study.

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REFERENCES

Archer, T. K., Cordingley, M. G., Wolford, R. G., & Hager, G. L. (1991) *Mol. Cell. Biol. 11*, 688.
Archer, T. K., Lefebvre, P., Wolford, R. G., & Hager, G. L. (1992) *Science 255*, 1573.
Archer, T. K., Lee, H.-L., Cordingley, M. G., Mymryk, J. S., Fragoso, G., Berard, D. S., & Hager, G. L. (1994) *Mol. Endocrinol. 8(5)*, 568.
Beato, M. (1989) *Cell 56*, 335.
Brenowitz, M., Senear, D. F., Shea, M. A., & Ackers, G. K. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8462.
Bresnick, E. H., John, S., Berard, D. S., Lefebvre, P., & Hager, G. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3977.
Bresnick, E. H., John S., & Hager, G. L. (1991) *Biochemistry 30*, 3490.
Bresnick, E. H., Bustin, M., Marsaud, V., Richard-Foy, H., & Hager, G. L. (1992) *Nucleic Acids Res. 20*, 273.
Buttinelli, M., Di Mauro, E., & Negri, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9315.
Chao, M. V., Gralla, J., & Martinson, H. G. (1979) *Biochemistry 18*, 1068.
Dixon, W. J., Hayes, J. J., Levin, J. R., Weidner, M. F., Dombroski, B. A., & Tullius, T. D. (1991) *Methods Enzymol. 208*, 380.
Dong, F., Hansen, J. C., & van Holde, K. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5724.
Drew, H. R., & Travers, A. A. (1985) *J. Mol. Biol. 186*, 773.
Felsenfeld, G. (1992) *Nature 355*, 219.
Fragoso, G., John, S., Roberts, M. S., & Hager, G. L. (1995) *Genes Dev. 9(15)*, 1933.
Gottesfeld, J. M. (1987) *Mol. Cell Biol. 7*, 1612.
Grunstein, M. (1990) *TIG 6*, 395.
Gunzburg, W. H., & Salmons, B. (1992) *Biochem. J. 283*, 625.
Hager, G. L. (1988) in *Breast Cancer: Cellular and Molecular Biology* (Lippman, M. E., & Dickson, R., Eds.) pp 267−281, Kluwer Academic Publishers, Boston.
Hager, G. L., & Archer, T. K. (1991) in *Structure and Function of Nuclear Hormone Receptors* (Parker, M. G., Ed.) pp 217−234, Academic Press, London.
Hager, G. L., Archer, T. K., Fragoso, G., Bresnick, E. H., Tsukagoshi, Y., John, S., & Smith, C. L. (1993) *Cold Spring Harbor Symp. Quant. Biol. 63*, 63−71.
Hansen, J. C., & Rickett, H. (1989) *Anal. Biochem. 179*, 167.
Hayes, J. J., Tullius, T. D., & Wolffe, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7405.
Hayes, J. J., Bashkin, J., Tullius, T. D., & Wolffe, A. P. (1991a) *Biochemistry 30*, 8434.
Hayes, J. J., Clark, D. J., & Wolffe, A. P. (1991b) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6829.
Horz, W., & Altenburger, W. (1981) *Nucleic Acids Res. 9*, 2643.
Li, Q., & Wrange, O. (1993) *Genes Dev. 7*, 2471.
Linxweiler, W., & Hörz, W. (1982) *Nucleic Acids Res. 10*, 4845.
Linxweiler, W., & Hörz, W. (1984) *Nucleic Acids Res. 12*, 9395.
Linxweller, W., & Hörz, W. (1985) *Cell 42*, 281.
Lomonossoff, G. P., Butler, P. J., & Klug, A. (1981) *J. Mol. Biol. 149*, 745.
Luisi, B. F., Xu, W. X., Otwinowski, Z., Freedman, L. P., Yamamoto, K. R., & Sigler, P. B. (1991) *Nature 352*, 497.
Lutter, L. C. (1977) *J. Mol. Biol. 117*, 53.
Lutter, L. C. (1978) *J. Mol. Biol. 124*, 391.
Meersseman, G., Pennings, S., & Bradbury, E. M. (1992) *EMBO J. 11*, 2951.
Muller, H. P., & Varmus, H. E. (1994) *EMBO J. 13*, 4704.
Nobile, C., Nickol, J., & Martin, R. G. (1986) *Mol. Cell Biol. 6*, 2916.

Pennings, S., Meersseman, G., & Bradbury, E. M. (1991) *J. Mol. Biol. 220*, 101.
Pennings, S., Meersseman, G., & Bradbury, E. M. (1992) *Nucleic Acids Res. 20*, 6667.
Perlmann, T., & Wrange, O. (1988) *EMBO J. 7*, 3073.
Pina, B., Barettino, D., Truss, M., & Beato, M. (1990a) *J. Mol. Biol. 216*, 975.
Pina, B., Brüggemeier, U., Beato, M. (1990b) *Cell 60*, 719.
Pina, B., Haché, R. J. G., Arnemann, J., Chalepakis, G., Slater, E. P., & Beato, M. (1990c) *Mol. Cell. Biol. 10*, 625.
Pina, B., Truss, M., Ohlenbusch, H., Postma, J., & Beato, M. (1990d) *Nucleic Acids Res. 18*, 6981.
Prunell, A. (1983) *Biochemistry 22*, 4887.
Prunell, A., & Kornberg, R. D. (1978) *Philos. Trans. R. Soc. London B, Biol. Sci. 283*, 269.
Pryciak, P. M., & Varmus, H. E. (1992) *Cell 69*, 769.
Pryciak, P. M., Sil, A., & Varmus, H. E. (1992) *EMBO J. 11*, 291.
Ramsay, N., Felsenfeld, G., Rushton, B. M., & McGhee, J. D. (1984) *EMBO J. 3*, 2605.
Rhodes, D. (1985) *EMBO J. 4*, 3473.
Richard-Foy, H., & Hager, G. L. (1987) *EMBO J. 6*, 2321.
Richmond, T. J., Finch, J. T., Rushton, B., Rhodes, D., & Klug, A. (1984) *Nature 311*, 532.
Roberson, A. E., Wolffe, A. P., Hauser, L. J., & Olins, D. E. (1989) *Nucleic Acids Res. 17*, 4699.
Satchwell, S. C., Drews, H. R., & Travers, A. A. (1986) *J. Mol. Biol. 191*, 659.
Simpson, R. T., & Stafford, D. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 51.
Sistare, F. D., Hager, G. L., & Simons, S. S. J. (1987) *Mol. Endocrinol. 1(9)*, 648.
Stein, A. (1989) *Methods Enzymol. 170*, 585.
Thoma, F., & Simpson, R. T. (1985) *Nature 315(6016)*, 250.
Truss, M., Bartsch, J., Hache, R. S., & Beato, M. (1993) *J. Steroid Biochem. Mol. Biol. 47*, 1.
von Holt, C., Brandt, W. F., Greyling, H. J., Lindsey, G. G., Retief, J. D., Rodrigues, J. D., Schwager, S., & Sewell, B. T. (1989) *Methods Enzymol. 170*, 431.
Wolffe, A. P., & Drew, H. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9817.
Wray, W., Boulikas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem. 118*, 197.
Zhang, X. Y., & Horz, W. (1984) *J. Mol. Biol. 176*, 105.

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