## Evidence that nucleosomes on the mouse mammary tumor virus promoter adopt specific translational positions

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## ABSTRACT

We have previously demonstrated that an array of six nucleosomes are phased on the mouse mammary tumor virus (MMTV) long terminal repeat (1,2). In this study, we devised a new assay to measure the translational positions of specific nucleosomes on the MMTV promoter. Nucleosome core particles were purified and shown to contain A and B nucleosomal DNA by Tag polymerase primer extension with nucleosome-specific primers. The 5' and 3' boundaries of A and B nucleosomes were measured by extending to the end of the core DNA with internal primers. This approach yielded results consistent with major translational positions of -23 to +123 and -221 to -75 for A and B nucleosomes, respectively. The micrococcal nuclease cleavage patterns of A and B nucleosome regions in isolated nuclei are conserved at basepair resolution in multiple murine cell lines containing either stable MMTV-reporter chimeras or endogenous proviruses. As the refined nucleosome positions place important transcription factor binding sites at the 3' edge of the B nucleosome and in the nucleosome A/B linker, we propose that linker histone depletion and chromatin unfolding may be required to expose these cis-elements during steroid hormone-induced transcription initiation.

## INTRODUCTION

The MMTV LTR has been widely used as a model steroid hormone-responsive element. We have previously shown that an array of six nucleosomes are phased on the LTR (1,2). Nucleosome positions were assigned from indirect end-labeling studies at a resolution of approximately +/-20 bp. Activation of the MMTV promoter by the hormone-bound GR results in endonuclease hypersensitivity on one of these nucleosomes (nuc-B) and in the nuc-B/nuc-A linker (1,3). As MMTV cis-elements are well characterized and are organized into a reproducible nucleosomal structure, this is an ideal system for asking how the

organization of DNA regulatory sequences into nucleosomes and higher order chromatin structures contributes to transcriptional regulation.

We have previously hypothesized that hormone-induced disruption of nuc-B structure (1,3,4) and H1 depletion (5) are necessary for NF-1 binding and assembly of a preinitiation complex on the MMTV promoter. Considering the general importance of a model that implicates positioned nucleosomes as specific repressors of transcription initiation, we have undertaken high resolution chromatin mapping experiments to address the following questions: (i) how precisely are nuc-A and nuc-B positioned, and (ii) where are transcription factor binding sites relative to nuc-A and nuc-B cores? In this report, we describe a new assay that utilizes the Taq polymerase and purified nucleosome core particles to measure the boundaries of nuc-A and nuc-B. We also identify MNase cleavage sites on both strands of the MMTV promoter, allowing us to refine previous nucleosome positions that were based on low resolution measurements. These refined positions place the NF-1 site at the 3' edge of nuc-B, OCT-1 sites in nuc-A/nuc-B linker, the TATA box at the 5' edge of nuc-A and the transcription intiation site within nuc-A.

## MATERIALS AND METHODS

## Cell culture and plasmids

Cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum. Cell lines 904.131 and 1361.5 were obtained by stable transfection of the chimeric constructs pM18 and pM23 into C127 and NIH3T3 cells, respectively (6,7). Plasmid pM18 contains the MMTV LTR driving the v-Ha-ras gene, and pM23 is a related construct in which the sv-40 polyadenylation signal and small tumor antigen splice site is inserted downstream of the v-Ha-ras coding sequence. Cell lines (904.131 and 1361.5) stably replicate pM18 and pM23 at approximately 200 and 125 copies, respectively. Cell line 1505 was previously derived from NIH3T3 cells by stable transfection with the LTR-v-Ha-ras construct, mp8al4 (8).

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Construct mp8al4 is integrated as a single copy into chromosomal DNA in 1505 cells. These cells also contain eight copies of integrated MMTV provirus (P. Lefebvre, unpublished observations).

#### Nuclei isolation and nuclease digestions

Nuclei were isolated from  $2 \times 10^8$  cells that had been treated for 1 h with 100 nM dex or ethanol vehicle. Cells were scraped into ice-cold PBS and were collected by centrifugation for 3 min at  $200 \times g$ . Cells were washed once by resuspension in PBS and recentrifugation. The washed cell pellet was resuspended in 10 mM Tris-HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 0.2% nonidet-P40, 5% sucrose, 1 mM DTT, 0.15 mM spermidine, 0.5 mM spermine at  $5 \times 10^7$  cells/ml, and cells were lysed by 10 strokes of a Dounce homogenizer (B pestle). This material was layered on a cushion of lysis buffer containing 10% sucrose and centrifuged at  $1600 \times g$  for 4 min. The supernatant was discarded, and the nuclei were washed by resuspension in 10 mM Tris-HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 1 mM DTT, 0.15 mM spermidine, 0.5 mM spermine  $(2.5 \times 10^7 \text{ nuclei/ml})$ , followed by recentrifugation at  $600 \times g$  for 3 min. Washed nuclei were resuspended in wash buffer, and 0.5 ml aliquots were used for MNase digestion. The digestions were initiated by addition of CaCl<sub>2</sub> to 1 mM and MNase (Worthington) from 40 to 480 units/ml. After an incubation for 5 min at 22°C, reactions were terminated with 0.5 ml of 10 mM Tris-HCl (pH 8.0), 25 mM EDTA, 1% SDS, 0.5 mg/ml proteinase K. Samples were incubated for at least 12 h at 37°C. Genomic DNA was purified by multiple extractions with phenol/chloroform, followed by ethanol precipitation.

#### Purification of nucleosome core particles

MNase-trimmed core particles were purified by the method of Ausio & Van Holde (9) as modified by Bresnick *et al.* (2). Aliquots from each fraction of a 5-25% sucrose gradient were analyzed by nondenaturing PAGE (6% gel), and nucleoprotein complexes were visualized by ethidium bromide staining. Fractions corresponding to the nucleosome core peak were pooled and concentrated to approximately 2 mg/ml protein with Centricon 10 microconcentrators (Amicon). Core DNA was purified by multiple rounds of phenol-chloroform extraction and used to analyze nucleosome boundaries.

#### **Oligonucleotide** primers

Oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer. Primers are referred to in the text by number, and correspond to the following positions relative to the MMTV RNA initiation site:

#343 (-105 to -129), 5'-AGCTCAGATCAGAACCTTTGATACC-3' #344 (-200 to -176), 5'-TTAAGTAAGTTTTTGGTTACAAACT-3' #471 (-278 to -252), 5'-GAACATTATTCTGCAAAAACTTATGGC-3' #399 (+160 to +139), 5'-GCTTCGGTACCAAACTGAAACC-3' #445 (-7 to +20), 5'-TAAACTTGCAACAGTCCTAACATTCAC-3' #446 (+89 to +70), 5'-ACCCTCTGGAAAGTGAAGGA-3' #4470 (-15 to -39), 5'-AATCAGCACTCTTTTATATCTTGGT-3'

#### Primer extension analysis of core particle and genomic DNA

Gel-purified oligonucleotide primer (5 pmoles) was labeled with [ $^{32}$ P]ATP and polynucleotide kinase according to standard procedures {816}. Genomic DNA (10  $\mu$ g for 1361.5 and 904.131 cells, 30  $\mu$ g for 1505 cells) or core particle DNA (1  $\mu$ g) was incubated in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM

MgCl<sub>2</sub>, 0.05% nonidet-P40, 0.05% Tween-20, 0.8 mM dATP, dCTP, dGTP, dTTP (or appropriate dideoxy NTP mixes), 0.4 pmoles of end-labeled primer  $(6-20 \times 10^6$  cpm), and 2.5 units of Amplitaq (Perkin Elmer Cetus) in a final volume of 30  $\mu$ l. Thirty cycles of primer extension were carried out using an annealing temperature of 2°C below the oligonucleotide melting temperature, calculated from its%GC content (10). Samples were extracted once with phenol/chloroform, ethanol precipitated, and analyzed on 8 or 10% polyacrylamide-urea sequencing gels. Gels were exposed to X-ray film for 12 h to 4 days.

## RESULTS

# Analysis of nuc-A and nuc-B translational positions by Taq amplification of core particle DNA

We have devised an assay to measure translational positions of specific nucleosomes. As outlined in Fig. 1, MNase-trimmed core particles were purified and MMTV nucleosome-specific oligonucleotide primers, complementary to regions well within specific nucleosomes, were annealed to the purified core DNA. Primer extension was performed with the Taq polymerase to the ends of the nucleosome core DNA, yielding a band that represents the nucleosome boundary. Parallel dideoxy sequencing reactions with MMTV plasmid DNA template allow one to assign the 5' and 3' bp limits of specific nucleosomes in a pool of total cell core particles. In addition to detecting a strong band that represents the end of the trimmed core, the assay also detects



Figure 1. Schematic outline of method to measure the translational positions of specific nucleosomes. Nucleosome core particles were prepared, and core DNA was used to analyze nuc-A and nuc-B translational positions as described in Materials and Methods. Rectangles, nucleosome cores; solid circles, histone H1.

nicks within the core that are generated during the digestion of nuclei with MNase and subsequent trimming of solubilized core particles.

As shown in Fig. 2A, end-labeled core DNA used for analysis of the nuc-B translational position has a mean size of  $140 \pm 6$  bp. Analysis of the 5' end of nuc-B (Fig. 2B) with the 343 primer reveals a strong band at -223 and a much weaker band at -231. As there is no extension beyond -231, and based on the predominance of the -223 band, it is likely that -223 is very close to the 5' boundary of nuc-B. A similar result was obtained with a primer that anneals to MMTV sequences from -125 to -146. The -223 position is also in good agreement with the



**Figure 2.** Determination of nuc-A and nuc-B translational positions by Taq polymerase primer extension of core particle DNA. Core particles were isolated from 904.131 cells by sucrose gradient centrifugation, and core DNA was purified by multiple phenol/chloroform extractions. Core DNA was end-labeled to determine the average size of the DNA fragments (panel A). The 5' (panel B) and 3' (panel C) boundaries of nuc-B were analyzed by primer extension with Taq polymerase (343 and 344 primers, respectively), as depicted in the figure. The 5' (panel D) and 3' boundaries (panel E) of nuc-A were analyzed by primer extension with Taq polymerase (446 and 445 primers, respectively), as depicted in the figure. Parallel extensions were performed with MNase-digested chromatin samples from 904.13 cells (chromatin). Positions of major bands were designated from parallel dideoxy A, C, G and T reactions. Representative dideoxy sequencing ladders are shown in panels A-E (M).

5' nuc-B boundary detected *in vitro* with an A-B reconstituted disome (4). Analysis of the 3' end of nuc-B (Fig. 2C) with the 344 primer reveals strong bands at -86, -84, -73 and -53, demonstrating that the 3' end of nuc-B is not downstream of -53 and consistent with positioning of nuc-B between -223 and -53. Assuming that nuc-B occupies a region of approximately 146 bp downstream of -223, this would yield a 3' end of -77, in line with the 3' measurement of -73.

In a second set of experiments, core particles with a mean DNA size of 149 + 1 - 6 bp were used to measure nuc-B boundaries and similar results were obtained [5' band, -226; 3' bands, -73, -53] (data not shown). As the -53 band persists even after more rigorous trimming, the resistance to additional trimming may reflect a specific structural feature of the 3' end of nuc-B, or alternatively, an unfavorable sequence for MNase cleavage.

Analysis of the 5' end of nuc-A (Fig. 2D) with the 446 primer reveals a strong band at -27. As shown in Fig. 2E, analysis of the 3' end of nuc-A with the 445 primer reveals a strong band



Figure 3. Primer extension analysis of MNase cuts in the nuc-A region of the MMTV promoter. Panel A—Indirect end-labeling analysis of MMTV chromatin in 1361.5 cells from (1) Panel B—Coding strand analysis. Genomic DNA from vehicle and dex-treated 1361.5 cells was analyzed by primer extension with Taq polymerase (399 primer) as depicted at the bottom of the figure. Panel C—Noncoding strand analysis. Genomic DNA from vehicle and dex-treated 1361.5 cells was analyzed by primer extension with Taq polymerase (445 primer) as depicted at the bottom of the figure. Specific treatment conditions are outlined at the top of the figure.



Figure 4. Primer extension analysis of the nuc-B region of the MMTV promoter. Panel A—Coding strand analysis of high copy number cell lines. Genomic DNA from vehicle and dex-treated 1361.5 cells or 904.131 cells was analyzed by primer extension with Taq polymerase (470 primer) as depicted at the bottom of the figure. Panel B—Coding strand analysis of a low copy number cell line. Genomic DNA from vehicle and dex-treated 1505 cells was analyzed by primer extension with Taq polymerase as depicted at the bottom of the panel. C—Non-coding strand analysis. Genomic DNA from vehicle and dex-treated 1361.5 cells was analyzed by primer extension with Taq polymerase as depicted at the bottom of the panel. C—Non-coding strand analysis. Genomic DNA from vehicle and dex-treated 1361.5 cells was analyzed by primer extension with Taq polymerase (471 primer) as depicted at the bottom of the panel. Specific treatment conditions are outlined at the top of each panel. The position of nuc-B is based on the general pattern of protection and does not imply exact 5' and 3' boundaries. Symbols: open arrows, bands that are enhanced in chromatin; half-filled arrows, bands that are unchanged; A, dideoxy A sequencing reaction; F or Free, deproteinized pM18 plasmid DNA digested with 2.5 units/ml of MNase.

at +126. The enrichment of the -27 and +126 bands demonstrates that nuc-A is positioned within the 153 bp region between -27 and +126.

#### Analysis of nuc-A and nuc-B translational positions by Taq amplification of genomic DNA from MNase digested nuclei

The indirect end-labeling experiments that initially defined the nucleosomal array on the MMTV LTR were performed by cleaving chromatin in isolated nuclei with MNase. We have extended this approach by perfoming a high resolution analysis of MNase digested chromatin to assign bp values for MNase cleavage sites. A representative indirect end-labeling analysis of nucleosome positions on the MMTV LTR from our previous work (1) is shown in Fig 3A. The cleavage pattern observed (180-200 bp repeats) is characteristic of DNA that is organized into an array of positioned nucleosomes.

A high resolution analysis of MNase cuts on the coding and noncoding strands of the nuc-A region of the MMTV promoter in 1361.5 cells is shown in panels B and C, respectively, of Fig. 3. A broad zone of protection against MNase cleavage is flanked by strong cuts at -27 and +108 (panel B), consistent with the resistance pattern expected for a histone octamer. At high concentrations of MNase (240 u/ml), internal cuts within the zone of protection become apparent. Panel C shows a similar region of protection, flanked on the 3' end by strong cuts at +109 and +124. These cuts flanking nuc-A are in accord with the core particle studies in Fig. 2 and our previous low resolution indirect end-labeling studies (1,2).

Comparison of the chromatin patterns from vehicle and dextreated cells reveals strong similarity, in agreement with our low resolution studies (1,2), albeit subtle hormone-dependent enhancements in cleavage occur at -27 and +107 on the coding strand (compare lanes 3 and 4 of Fig. 3A). The apparent hormone-dependent region of protection between -16 and +17(Fig. 3B, lanes 3 and 4) has not been found to be reproducible.

We have performed similar experiments with the nuc-B region, although a higher degree of internal cleavage within the nuc-B core is observed compared to nuc-A. Fig. 4 shows a representative analysis of MNase cuts in this region. Panels A and B represent the coding strand of the nuc-B region of the MMTV promoter (panel A; 1361.5 cells, pM23 MMTV-ras fusion, or 904.13 cells, pM18 MMTV-ras fusion: panel B; 1505 cells ,several endogenous MMTV proviruses and one MMTVras fusion). Panel C represents the non-coding strand of the nuc-B region (1361.5 cells, pM23 MMTV-ras fusion). Similar to the results shown in Fig. 3 for nuc-A, there is also good general protection against MNase cleavage throughout the entire nuc-B region. This broad zone of protection is consistent with the specific positioning of a nucleosome, as demonstrated in Fig. 2 with purified nucleosome cores.



Figure 5. Model of specific nucleosomal organization of the MMTV promoter. Nucleosome boundaries from high resolution MNase mapping data were used to assign positions of dyad symmetry (+). Regions of 146 bp were designated based on the nucleosome dyads and are shown below the schematic. Transcription factor binding sites are indicated by hatched boxes: GRE-d, distal GRE; GRE-p, proximal GREs; NF-1, nuclear factor 1; OCT1, octamer binding protein 1; TFIID, transcription factor IID.

Regions of strong MNase sensitivity in chromatin are, in some cases, also preferential cleavage sites with deproteinized DNA (half-filled arrows), in agreement with the observations of Keene and Elgin (11). Significant differences exist, however, between the chromatin and free DNA cleavage patterns, and these differences are indicated by open arrows (sites that are repressed in chromatin) and filled arrows (sites that are enhanced in chromatin). For example, coding strand cuts at -84, -103, -148, and -166 are stronger in chromatin than in free DNA, and cuts at -77, -85, -86, -104, -111, -127, and -150 are strongly repressed in chromatin relative to free DNA (compare lanes 5 and 8 of Fig. 4A). A hallmark of the specific chromatin pattern is a pair of strong cuts at -84 and -86, whereas, free DNA is characterized by strong cuts at -85 and -86 and a relatively weaker cut at -84. Cuts at -85 and -229/-230 on the non-coding strand flank a core region of protection (Fig. 4C). The presence and relative intensities of chromatin-specific bands are identical in the three cell lines presented here, as well as in 1470.2 cells which contain the pM25 MMTV-CAT fusion (data not shown). The MNase cleavage patterns are very reproducible and are consistent with a model in which nuc-A and nuc-B exist each in a major translational position.

#### DISCUSSION

In this study, we described a new assay for determining the translational positions of specific nucleosomes and used it to assign boundaries for nuc-A and nuc-B on the MMTV promoter. In addition, we assigned bp values for MNase cleavage sites on the promoter and demonstrated that the specific cleavage pattern is conserved in multiple cell types (Fig. 4). The specific and reproducible nucleosome positioning on the MMTV promoter suggests that a nonrandom chromatin organization can be advantageous to the cell and may be important for transcriptional regulation.

The high resolution chromatin mapping experiments presented in this manuscript demonstrate that nuc-B and nuc-A adopt major translational positions between -223 to -73 and -27 to +126, respectively. To obtain estimates of the precise nucleosome core boundaries from these experimental measurements, we used the midpoint obtained from the external limits mentioned above to assign a dyad axis of symmetry (nuc-B, -148; nuc-A, +50). Assuming a core size of 146 bp, we used the dyad assignments to calculate core boundaries of -221 to -75 and -23 to +123 for nuc-B and nuc-A, respectively (Fig. 5). These results confirm and extend our previous low resolution analysis of MMTV chromatin, in which nucleosome positions were designated with an accuracy of approximately  $\pm 20$  bp (1,2) and were shown to be identical in multiple cell lines containing chimeric MMTV LTR-reporter constructs.

The assay that we have described to measure the translational positions of nuc-A and nuc-B with Taq polymerase and purified core particles should be generally applicable to a variety of other systems. A necessary prerequisite for such an endeavor is careful indirect end-labeling data for designing internal nucleosome-specific primers. Although our studies utilized multi-copy templates, we estimate that the assay could be adapted for analysis of single copy genes by increasing the amount of core DNA analyzed and, if necessary, incorporating a ligation step with a common linker (12) to allow for exponential amplification of the target nucleosomal DNA.

A consideration of the precise mechanism by which specificity and conservation of MMTV chromatin organization arises must involve two distinct but interacting aspects of nucleosome positioning, translational and rotational positioning [for reviews see (13,14)]. In vitro reconstitution experiments with nuc-B region DNA fragments and purified histones or core particles have provided evidence for a single specific rotational position (2,15,16). On the other hand, analysis of the boundaries of reconstituted nuc-B monosomes (2,15,16) and a nuc-B/nuc-A disome (4) has revealed multiple ExoIII stops that vary by 10 bp or a single turn of the DNA helix (2,4,15,16). These results imply that nucleosomes reconstituted on MMTV fragments in vitro exist in multiple translational positions. For the case of the reconstituted nuc-A/nuc-B disome, the nuc-B 5' boundary was reported to be at position -221 (4), in close agreement with the core particle results presented here. It should be noted that the positions we report here represent the major detectable position in vivo. Multiple minor frames consistent with the multiple positions observed in vitro would probably not be detected.

Analogous to the ExoIII results with reconstituted MMTV nucleosomes, it was recently reported that a reconstituted 5S ribosomal RNA gene monosome adopts multiple translational frames, despite a single rotational position (17). The studies presented here suggest that additional constraints are exerted on MMTV nucleosomes by the cell to select for a single translational position *in vivo*. *In vivo* DNaseI footprinting experiments are in progress to determine if the specific nuc-B rotational position observed *in vitro* (2,15,16) is also favored by the cell. Preliminary characterization of the DNaseI cleavage pattern of MMTV chromatin in isolated nuclei at bp resolution (unpublished data) reveals a more complex pattern than the clear 10 bp periodic cleavage observed with *in vitro* reconstituted nuc-B monosomes (2,15,16).

A number of distinct models have been proposed to account for the precise positioning of nucleosomes. These include, (i) boundaries imposed by specific DNA-binding proteins (18-21); (ii) constraints exerted by higher order chromatin folding (22); (iii) intrinsic properties of the DNA, e.g. bendability (23). Pina *et al.* (24) recently demonstrated that a DNA fragment spanning the MMTV promoter exhibited characteristics of bent DNA in a circular permutation assay. The MMTV LTR should provide a particularly good model system for ascertaining the relative contribution of each of these factors to constraining nucleosomes

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on a well-defined transcriptional regulatory element. One can ask whether these determinants of positioning constitute a level of transcriptional control independent of requisite transacting factors. Such an analysis of nucleosome positioning determinants may be greatly facilitated by the assay described herein using purified core particles and Taq polymerase.

In conclusion, high resolution studies of nucleosome positions on the MMTV promoter demonstrate that the NF-1 binding site is at the edge of nuc-B, OCT-1 binding sites are within the nuc-B/nuc-A linker, the TATA box is at the 5' edge of nuc-A and the transcription initiation site is within nuc-A. As H1 binds to linker regions of chromatin (25), and we have recently shown that H1 is bound to the MMTV promoter (5), our data are consistent with a model in which NF-1 and OCT-1 binding sites are obscured either directly by H1, by an H1-nucleosome complex (4) or indirectly by folding of MMTV chromatin.

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