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Hormone activation induces nucleosome positioning *in vivo*

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The mouse mammary tumor virus (MMTV) promoter is induced by glucocorticoid hormone. A robust hormone- and receptor-dependent activation could be reproduced in *Xenopus laevis* oocytes. The homogeneous response in this system allowed a detailed analysis of the transition in chromatin structure following hormone activation. This revealed two novel findings: hormone activation led to the establishment of specific translational positioning of nucleosomes despite the lack of significant positioning in the inactive state; and, in the active promoter, a subnucleosomal particle encompassing the glucocorticoid receptor (GR)-binding region was detected. The presence of only a single GR-binding site was sufficient for the structural transition to occur. Both basal promoter elements and ongoing transcription were dispensable. These data reveal a stepwise process in the transcriptional activation by glucocorticoid hormone.

Keywords: chromatin structure/glucocorticoid receptor/MMTV promoter/nucleosome positioning/*Xenopus* oocyte

Introduction

All eukaryotic cells have their DNA packaged into a protein–DNA structure, chromatin. The basic subunit of chromatin is the nucleosome (Luger *et al*., 1997), which can be located at specific DNA segments in the eukaryotic genome (Simpson, 1991). Translational nucleosome positioning depends on local variations in DNA curvature, helical periodicity and/or boundary effects. When present in gene regulatory regions, nucleosomes can act as a barrier to the process of transcriptional initiation (Han *et al*., 1988; Perlmann and Wrange, 1991) in a gene-specific manner (Wyrick *et al*., 1999). This repressive effect of chromatin is modulated at specific loci by the rapid remodeling of the chromatin structure during gene activation. One well studied example of transcription activation-dependent chromatin remodeling is the mouse mammary tumor virus (MMTV) promoter. This promoter is strongly induced by glucocorticoid hormone. Activation is associated with the appearance of a DNase I-hypersensitive site (Zaret and Yamamoto, 1984; Truss *et al*., 1995) over the glucocorticoid response element (GRE). Micrococcal nuclease (MNase) and methidiumpropyl-EDTA·iron(II) complex (MPE) digestion of the MMTV long terminal repeat (LTR) *in situ* initially revealed that this regulatory DNA segment harbors six translationally positioned nucleosomes and that nucleosome B, positioned over the GRE, undergoes an activation-dependent remodeling (Richard-Foy and Hager, 1987). Further mapping experiments at high resolution showed that nucleosomes, although not precisely positioned, displayed a clustered distribution (Fragoso *et al*., 1995). Interestingly, despite the drastic increase in transcription activation and the occurrence of a glucocorticoid-dependent DNase I-hypersensitive site (see above), careful *in vivo* analyses have failed to detect any hormone-induced changes at the level of nucleosomal organization for the MMTV LTR stably incorporated in tissue culture cells (Fragoso *et al*., 1995; Truss *et al*., 1995). In these *in vivo* studies, the positioned nucleosome ladder seemed to remain unchanged independent of the transcriptional status of the promoter.

In vivo footprinting experiments showed that basal transcription factors, such as nuclear factor 1 (NF1) and TATA-box binding factor (TFIID), do not interact with their cognate target sites in the MMTV promoter unless a hormone-induced chromatin remodeling event has taken place (Cordingley *et al*., 1987; Archer *et al*., 1992; Truss *et al*., 1995). This suggested a role for chromatin in keeping the inactive promoter in a closed configuration (Han *et al*., 1988; Perlmann and Wrange, 1991). As a direct implication, chromatin rearrangements should occur to permit the transition towards an active state. The discovery that transcription coactivators had histone acetyltransferase, histone deacetylase and nucleosome remodeling properties, as reviewed in Kingston and Narlikar (1999), further provided the possible molecular players in this process. However, structural aspects of *in vivo* chromatin remodeling during gene activation remain obscure.

Xenopus oocytes represent an attractive *in vivo* system to follow these issues. Estrogen- (Theulaz *et al*., 1988), glucocorticoid- (Perlmann and Wrange, 1991) and thyroid hormone-dependent (Wong *et al*., 1998) gene regulation occur in *Xenopus* oocytes merely by expression of the appropriate receptor protein(s) and by injection of a DNA reporter plasmid. Here, we revealed a robust glucocorticoid-dependent and transcription-coupled chromatin remodeling over the GRE of the MMTV LTR. This remodeling was homogeneous and, in contrast to previous results in tissue culture cells, it involved major rearrangements at the nucleosome level. The hormone-induced chromatin remodeling of the MMTV promoter resulted in induction of translational positioning of initially randomly

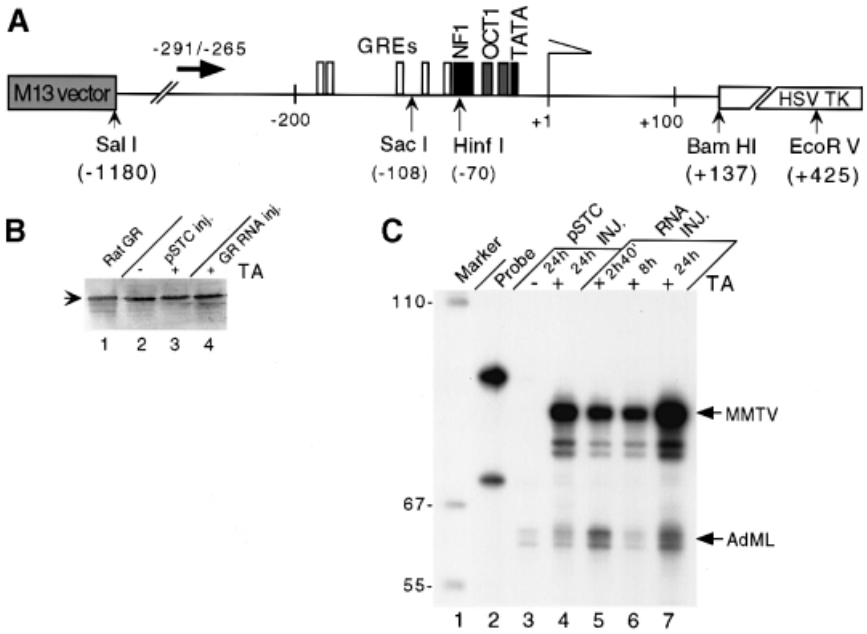

Fig. 1. Reconstitution of glucocorticoid regulation in *Xenopus* oocytes. (**A**) The reporter DNA construct, the pMTV:M13 coding vector with the primer used for primer extension analysis of the *Sac*I *in situ* accessibility assay (solid black arrow) and the restriction enzyme cleavage sites that are referred to in the text. White boxes, GRE hexanucleotide elements; black box, NF1 site; light gray box, OCT 1 site; and dark gray box, TATA-box sequence. (**B**) GR expression in oocytes. Western blot of SDS–PAGE: lane 1, GR prepared from rat liver (Perlmann and Wrange, 1988); lanes 2 and 3, one *Xenopus* oocyte equivalent was analyzed 24 h after injection of 5 ng of pSTC GR 3-795 expression vector; lane 4, one oocyte equivalent injected with 5 ng of *in vitro* transcribed GR RNA 24 h before analysis. Hormone (TA, 1 μM) was added as indicated. (**C**) Hormone-dependent MMTV transcription in *Xenopus* oocytes. Transcription analysis by S1 nuclease protection of MMTV and the AdML promoter. Oocytes in groups of five were injected with 1 ng of pMTV:M13 coding vector ssDNA and 0.25 ng of pAdML reference and either 5 ng of GR expression vector (pSTC GR 3-795) (lanes 3 and 4) or 5 ng of *in vitro* transcribed GR RNA (lanes 5–7). After 24 h, hormone (TA, 1 μM) was added to oocyte culture media and oocytes were harvested for RNA analysis at the time indicated.

organized nucleosomes. This was independent of basal promoter elements and of ongoing transcription, but required a high affinity glucocorticoid receptor (GR)-binding site, highlighting the distinct steps involved in hormone activation *in vivo*. We conclude that nucleosome positioning in the MMTV LTR is not functionally required to achieve hormone-dependent induction but is a consequence of the induction event.

Results

Chromatin assembly and glucocorticoid hormone induction in Xenopus oocytes

The MMTV LTR was fused to the herpes simplex virus thymidine kinase (HSVTK) gene coding sequence (Buetti and Kuhnel, 1986) and propagated in the M13 filamentous phage (Figure 1A). It was used for intranuclear *Xenopus* oocyte injections in single-stranded (ss) form. This results in replication-coupled chromatin assembly of the injected ssDNA (Almouzni and Wolffe, 1993), which leads to formation of naturally spaced chromatin. This can be monitored after MNase digestion by the appearance of DNA fragments whose lengths are multiples of the size corresponding to a nucleosome repeat length (Figure 2B).

GR protein is required to elicit a hormone response in *Xenopus* oocytes (Perlmann and Wrange, 1991). Full-length GR protein was provided either by nuclear injection of a cytomegalovirus (CMV) promoter-driven rat GR cDNA expression vector (pSTC GR 3-795), or by cytoplasmic injection of *in vitro* synthesized rat GR RNA. The presence of full-length GR protein was verified by immunoblotting (Figure 1B). The accumulation of the MMTV-specific mRNA was assayed by S1 nuclease protection analysis. The MMTV promoter was virtually silent in the absence of hormone (Figure 1C). In contrast, a strong induction was observed after addition of 1×10^{-6} M synthetic glucocorticoid hormone triamcinolone acetonide (TA) to the oocyte culture medium (Figure 1C). This MMTV promoter-driven transcription was already detectable after 2 h (data not shown). *Xenopus* oocytes translate injected RNA efficiently (Colman, 1984). The injection of *in vitro* transcribed GR RNA gave rise to a stronger transcriptional response than that obtained using the GR expression vector strategy (compare lanes 4 and 7 in Figure 1C). As a consequence, GR was usually provided by injection of GR RNA in subsequent experiments.

Glucocorticoid-induced chromatin remodeling in the MMTV LTR

To follow possible chromatin changes upon hormone induction, *Xenopus* oocytes were injected with ssMMTV reporter DNA and GR expression vector and incubated with TA (1 μM) for 24 h. The oocytes were then homogenized and digested with increasing amounts of DNase I. Digestion products were analyzed by an indirect end-labeling assay (Wu, 1989). These experiments revealed several glucocorticoid-dependent DNase I-hypersensitive segments within the MMTV LTR. The strongest DNase I hypersensitivity was distributed around position

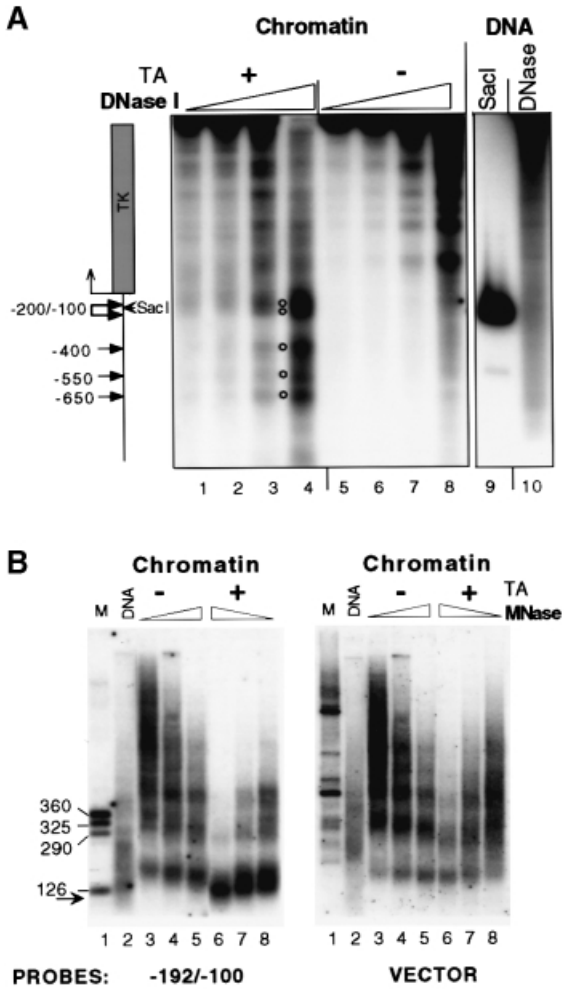

Fig. 2. Chromatin structure of the MMTV promoter. (**A**) Hormone-dependent DNase I-hypersensitive sites are located in the MMTV LTR. Groups of 12 oocytes were injected with 1 ng of pMTV:M13 coding ssDNA, 5 ng of dsDNA for pSTC GR 3-795 and 0.25 ng for pAdML reference (lanes 1–8). After overnight incubation, hormone was added (TA; 1 μM) (lanes 1–4) or not added (lanes 5–8) and oocytes were harvested after 24 h for the DNase I hypersensitivity assay. Lane 9, internal molecular weight marker showing the position of the *Sac*I restriction enzyme cut. Lane 10, naked dsMMTV promoter DNA digested with DNase I. (**B**) MNase *in situ* digestion shows hormone-dependent disruption of the canonical nucleosome structure in the vicinity of GRE elements. Groups of 10 oocytes were injected. The next day, hormone (TA; 1 μM) was added as indicated and oocytes were harvested after 24 h for MNase digestion. DNA was resolved in an agarose gel, transferred and hybridized with a labeled MMTV promoter probe encompassing region –192/–100, and then washed and rehybridized with an M13 vector probe. Lane 1, internal DNA marker; lane 2, naked dsMMTV promoter DNA digested with MNase. The arrow shows a subnucleosomal particle ~120 bp DNA fragment revealed only after hybridization with specific probe.

–200/–100, which includes the MMTV GRE, –185/–79 (compare Figure 2A, lane 4, labeled with a double circle, with lane 8 non-hormone-treated oocytes, and the location of the *Sac*I restriction site at –108, lane 9). There were additional hormone-dependent DNase I-hypersensitive regions with a lower intensity further upstream within the MMTV LTR. These were distributed around three positions, –400, –550 and –650 (Figure 2A, lane 4, open circles). The DNase I hypersensitivity at the –200/–100 position, however, was always the most prominent, and the extent of DNase I hypersensitivity correlated with the extent of MMTV transcription, as measured by S1 nuclease analysis (data not shown).

MNase digestion was used to examine in more detail the effects of hormone activation on chromatin structure in the vicinity of the GRE (–185/–79) (Payvar *et al*., 1983; Buetti and Kuhnel, 1986). Injected oocytes were homogenized and digested with MNase. Isolated DNA was resolved on an agarose gel, blotted and probed with a short MMTV promoter fragment –192/–100, covering the strongest DNase I-hypersensitive area. The substantial alteration of the canonical MNase ladder indicated that hormone activation leads to drastically increased MNase cutting of the DNA in the vicinity of the GRE region (Figure 2B left, compare lanes 3–5 with 6–8). Unexpectedly, the mononucleosome fraction at 146–185 bp, which was present in the inactive promoter, was replaced by an unusual subnucleosomal particle protecting a DNA fragment of ~120 ± 10 bp, in the active promoter (Figure 2B, left, compare lane 5 with lane 6). The relative resistance of this subnucleosomal particle to MNase digestion and its discrete migration on the agarose gel suggest that it represents a defined DNA–protein complex. Importantly, the signal corresponding to this subnucleosomal particle reflects the fact that chromatin remodeling involves the vast majority of the MMTV DNA copies. Reprobing the filter of the MNase *in situ* digested chromatin with M13 vector DNA as probe (Figure 2B, right) showed that the hormone-induced subnucleosomal particle was not present in the vector DNA (compare lanes 6–8 in the left and right panels). There is, however, a slight but clearly detectable hormone-dependent increase in MNase digestion in the vector DNA (Figure 2B, right, compare lanes 3–5 with lanes 6–8). We attribute this to chromatin 'domain' effects of the strong transcriptional response.

We conclude that transcription activation of the MMTV promoter results in (i) increased accessibility of DNA in the vicinity of the GRE segment; (ii) reorganization of the DNA harboring the GRE into a subnucleosomal protein–DNA complex that protects ~120 ± 10 bp of DNA; and (iii) virtually all copies of the MMTV template undergoing a similar nucleosome remodeling event in the vicinity of the GRE region.

Chromatin remodeling can be followed by *in situ* digestion of DNA with an appropriate restriction enzyme that cuts within the remodeled chromatin region (Archer *et al*., 1992; Truss *et al*., 1995; Fragoso *et al*., 1998). We used the *Sac*I restriction enzyme, which cuts the MMTV promoter at position –108 within the GRE segment. For quantitation, we carried out a primer extension analysis with a primer annealing to the DNA strand that is synthesized in the oocyte after ssDNA injection. In this way, molecules assembled into chromatin are specifically revealed. A distinct increase in *Sac*I cutting 1 h after hormone addition was observed and it reached a plateau after 3–6 h (Figure 3A). In addition, a significant stimulation of both chromatin remodeling (Figure 3B) and MMTV transcription (Figure 3C) was detected at a hormone concentration of 10 nM. This illustrates the parallel between chromatin remodeling and accumulation of MMTV RNA. Both hormone-dependent *Sac*I cutting and MMTV transcription were dependent on the presence of GR, which was provided here by GR RNA injection (see

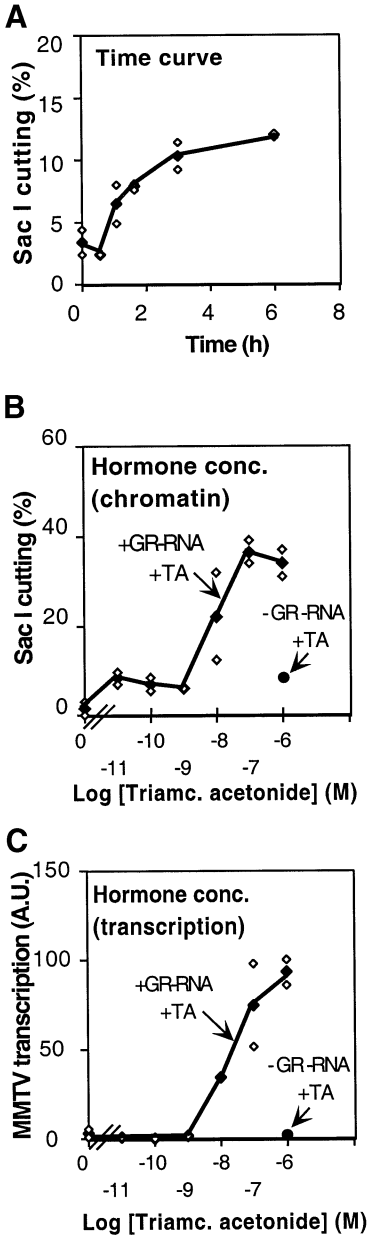

Fig. 3. Evaluation of the effect of time and hormone concentration on hormone-induced chromatin remodeling. (**A**) Oocytes were injected with 10 ng of GR RNA and 1 ng of ssDNA pMTV:M13 coding strand, and 0.25 ng of pAdML for reference. After overnight incubation, oocytes were divided into 12 groups of five oocytes each; 1 μM TA was added at various times. Oocytes were homogenized and two-thirds taken for *Sac*I *in situ* accessibility assay and one-third for RNA analysis (not shown). White diamonds signify each individual analysis as quantified by PhosophorImager, and black diamonds the mean value for each double sample. (**B**) Oocytes were injected with DNA and GR RNA (+GR-RNA) or with DNA only (–GR-RNA) and the next day divided into 16 groups with six oocytes in each and treated with the indicated concentrations of hormone (TA) for 9 h and then homogenized. Two-thirds was taken for *Sac*I and one-third for RNA analysis. Symbols as in (A). Log [TA] is given on the abscissa. (**C**) Quantitaion of MMTV RNA relative to AdML RNA of the experiment described in (B) using S1 nuclease protection assay and PhosphorImager analysis, arbitrary units (A.U.). Symbols as in (A).

inset right part of Figure 3B and C for oocytes not injected with GR RNA). We conclude that glucocorticoid hormone-induced chromatin remodeling (quantified by a *Sac*I *in situ* cutting assay) and transcription are closely correlated in the MMTV promoter.

Hormone activation induces translational nucleosome positioning in the MMTV LTR

The presence of many sequence-specific MNase cut sites in the MMTV LTR (Richard-Foy and Hager, 1987) made the use of this enzyme inappropriate to determine nucleosome positioning over these sequences. Thus, we used the chemical nuclease MPE, which has a strong preference for internucleosomal regions and that, due to its small size, shows almost no sequence specificity in DNA cleavage (Richard-Foy and Hager, 1987; Truss *et al*., 1995).

Figure 4A and B shows the MPE cleavage pattern of the MMTV promoter. Surprisingly, we observed no obvious nucleosomal pattern in the inactive MMTV promoter (Figure 4A and B, lanes 2 and 3). After hormone-induced transcription activation, however, a distinct cleavage pattern was seen, suggesting a strong nucleosome positioning over the entire MMTV LTR (compare lanes 2 and 3 with lanes 4 and 5 in Figure 4A and B). The nucleosome positioning revealed here by MPE digestion experiments in hormone-treated *Xenopus* oocytes coincides with that shown previously for the MMTV promoter stably incorporated into bovine papilloma virus (BPV)-based episomal vector constructs in tissue culture cells (Richard-Foy and Hager, 1987; Truss *et al*., 1995). In agreement with these results, our MPE cleavage pattern suggests that at least six nucleosomes are positioned over the MMTV LTR. Our interpretation of this MPE-induced pattern with respect to nucleosome positioning is shown (Figure 4A and B, diagram on the right). We have termed these positioned nucleosomes A–F following the previously used nomenclature (Richard-Foy and Hager, 1987). Contrary to previous findings, however, we did not observe any significant translational nucleosome positioning in the inactive MMTV promoter.

To determine to what extent the observed MPE cleavage pattern was caused by nucleosome positioning, we used an alternative strategy to map the borders of the putative dinucleosome that covers the –425/–60 segment of the MMTV promoter. This was achieved by isolating dinucleosomal DNA (360–370 bp) from an agarose gel after MNase digestion and determining its borders by restriction enzyme cutting. We reasoned that the agarose gel size selection would reduce the influence of any local sequence specificity of MNase cutting. Oocytes were processed for MNase digestion. Dinucleosomal DNA was isolated and cleaved with either *Hin*fI or *Rsa*I restriction enzymes (Figure 4D; the diagram on the right side displays the locations of restriction sites). The resulting DNA samples were resolved in a 3.5% agarose gel, blotted and hybridized with an α-^{32}P-labeled MMTV promoter probe that encompassed the –415/–100 segment of MMTV DNA. The hormone-activated MMTV promoter cleavage with *Hin*fI resulted in conversion of the 360–370 bp dinucleosome DNA fragment into a discrete 340 bp fragment, while cleavage with *Rsa*I generated distinct bands of 310 and 320 bp (Figure 4C, lanes 5 and 6). In contrast, digestion of the dinucleosomal DNA from the inactive MMTV promoter (not treated with hormone) with either *Hin*fI or *Rsa*I resulted in several bands ranging from 240 to 370 bp in size (Figure 4C, lanes 2 and 3). This result

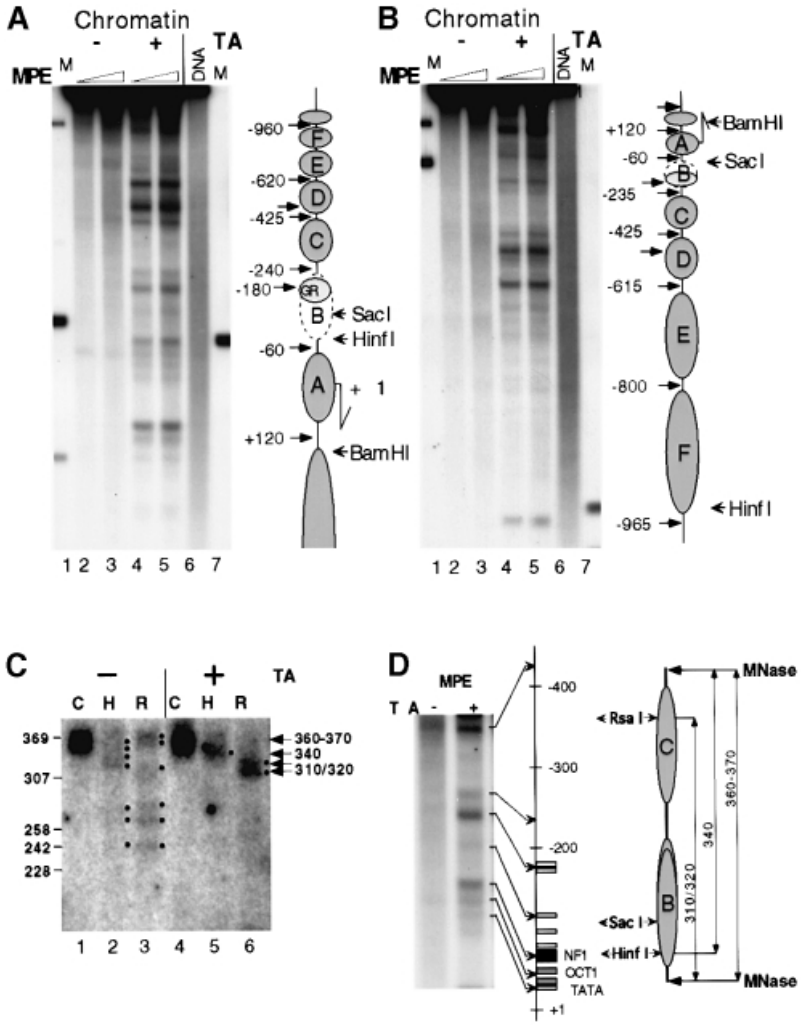

Fig. 4. Hormone-induced nucleosome positioning analyzed by MPE and MNase digestion *in situ.* (**A** and **B**) Transcriptional activation leads to establishment of nucleosome positioning along the MMTV promoter. Injected oocytes were analyzed after 24 h of hormone treatment. MPE digestion was performed for 3 min (lanes 2 and 4) and 10 min (lanes 3 and 5). Isolated DNA was digested with *Sal*I and *Eco*RV, resolved on agarose, blotted and hybridized first with a random-primed labeled fragment adjacent to the *Eco*RV site (*Eco*RV–*Sac*I fragment in A) and then stripped and reprobed with *Sal*I–*Rsa*I (B). Lanes 1 and 7, internal molecular weight markers (see map to the right); lanes 2–5, MPE digestion of injected oocytes, treated (lanes 4 and 5) and not treated (lanes 2 and 3) with hormone; lane 6, naked dsMMTV promoter DNA digested with MPE. To the right in (A) and (B) is a schematic summary of MPE cuts along the MMTV LTR with putative nucleosome positions. (**C**) Mapping of dinucleosome borders suggests that nucleosomes are translationally positioned along the MMTV promoter only after activation of transcription. Groups of 10 oocytes were hormone treated as in (A) and (B) and MNase digested as in Figure 2B (lanes 3 and 8). DNA was isolated and resolved in a 4% NuSieve GTG agarose gel together with size markers. The band corresponding to dinucleosome DNA (360–370 bp in length) was excised from the gel, DNA was eluted and analyzed as a control (lanes 1 and 4) or digested either with *Hin*fI (lanes 2 and 5) or *Rsa*I (lanes 3 and 6). DNA was resolved in a 1% SeaKem GTG + 2.5% NuSieve GTG agarose gel, blotted and hybridized with a random-primed probe encompassing region –415/–100. Black dots outline the DNA bands revealed by hybridization. (**D**) Chromatin organization of the MMTV promoter as revealed by MNase and MPE mapping. A magnified section of lanes 3 and 4 in (A) is shown together with a schematic presentation of the MMTV LTR and the restriction enzyme cleavage sites. All symbols are as in Figure 1A. The positions of the nucleosomes (on the right) are based on the results in (A–C). The co-localizations of MPE cuts with internucleosome linkers and/or factor-binding sites are indicated by arrows.

is in agreement with the MPE data, and the results taken together strongly suggest that the hormone-dependent activation of the MMTV promoter induces a precise nucleosome positioning of initially randomly organized nucleosomes. The additional MPE cut sites, which do not coincide with the location of the nucleosome linkers, could possibly reflect transactivating factors binding to DNA (Figure 4D, e.g. the –180 cut site, which coincides with a strong GR-binding site). These cut sites are clustered within and proximal to the nucleosome B segment where most transactive factors bind. Overall, the nucleosome B segment displays hormone-dependent hypercutting. This is in contrast to the nucleosome C segment, which shows hormone-induced MPE protection, probably reflecting translational positioning and the relative lack of factor binding in this region (Figure 4A, B and D).

Hormone-induced nucleosome positioning depends on the presence of GR-binding site(s) but not on OCT1, NF1 or TATA-box elements

To evaluate the influence of different promoter elements and their cognate transactivating factors on hormone-dependent chromatin remodeling, we created three MMTV LTR deletion mutants. These mutants and the wild type are represented in Figure 5A. Their hormone-dependent transcriptional activity was 10%, 1% and non-detectable for the

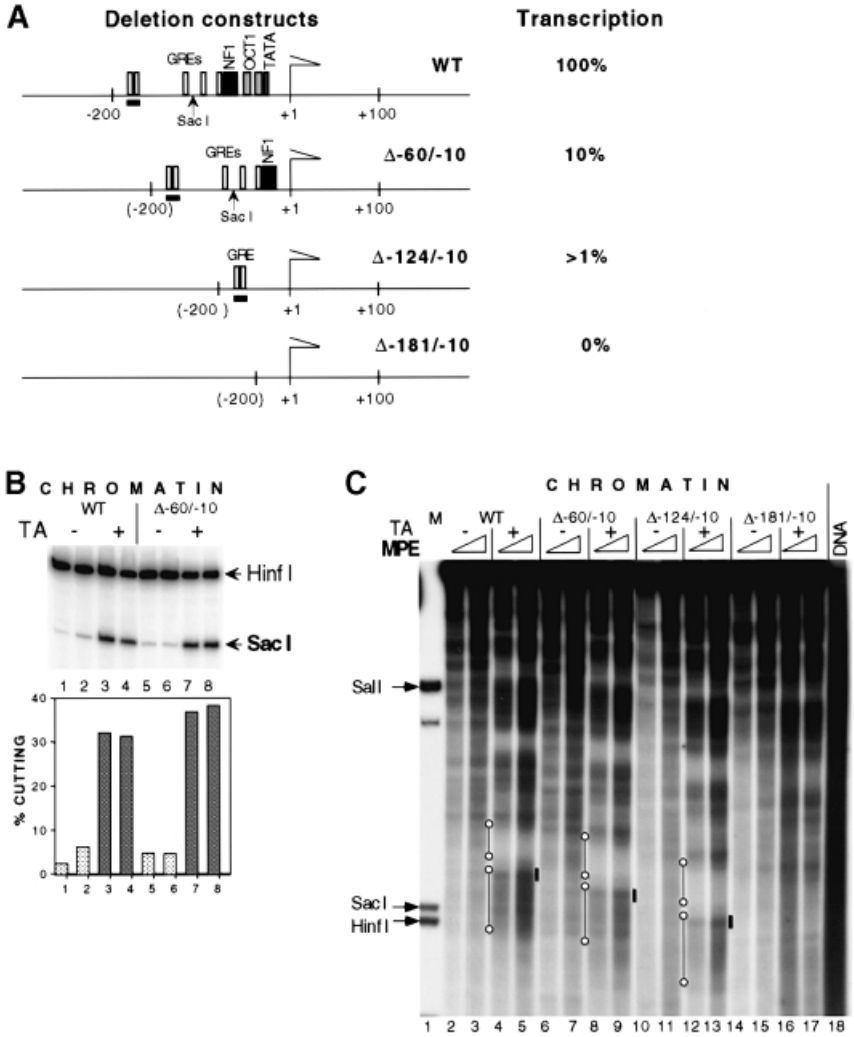

Fig. 5. Nucleosome remodeling and establishment of nucleosome positioning are dependent on GR binding but not on other basal promotor elements. (**A**) Maps of MMTV deletion mutants. Names of mutants signify the base pairs that were deleted relative to the transcription initiation start (+1). The strong GRE site at position –185/–171 in the wild type and the corresponding site in Δ–60/–10 and Δ–124/–10 mutants are underlined. Hormone-dependent transcriptional efficiency relative to wild type, as measured by S1 nuclease protection, is given on the right. (**B**) Nucleosome remodeling in the vicinity of the GRE elements in wild type (lanes 1–4) and Δ–60/–10 mutant (lanes 5–8) as revealed by *Sac*I restriction enzyme accessibility assay. Groups of five oocytes were subjected to the *Sac*I restriction enzyme accessibility assay. Arrows show specific bands generated by *Sac*I and *Hin*fI. The diagram below shows *Sac*I cutting as a percentage of total DNA. (**C**) MPE analysis. See Figure 4A legend for details. Lane 1, internal molecular weight marker, showing the positions of *Hin*fI and *Sac*I restriction enzyme cuts. Lanes 2–17, wild-type or mutant ssDNA injected as indicated. Lane 18, naked dsMMTV promoter DNA digested with MPE. Solid black lines mark the position of the strong GRE elements at –185/–171 in the wild type and in Δ–60/–10 and Δ–124/–10 mutants. Open circles connected with a black line mark the hormone-induced positioning of nucleosomes C and B (from top to bottom).

Δ–60/–10, Δ–124/–10 and Δ–181/–10 mutants, respectively, relative to the wild type (data not shown). None of the mutants displayed constitutive MMTV expression.

We analyzed the MMTV chromatin structure of the Δ–60/–10 mutant using two assays: MNase digestion and *Sac*I *in situ* cutting. In the first assay, a similar hormone-dependent increase in MNase cutting was revealed in both the mutant and wild-type promoter (not shown). The second assay further confirmed that these two promoters could respond to the hormone in a similar way at the structural level. The *Sac*I cutting profiles were superimposable (Figure 5B). MPE digestion experiments showed strong hormone-dependent nucleosome positioning along the MMTV LTR, and hormone-dependent hypercutting, indicating chromatin remodeling, around the GRE segment within the nucleosome B region in the wild-type MMTV promoter as well as in the Δ–60/–10 and Δ–124/–10 mutants (Figure 5C). As observed earlier (Figure 4A and B), the nucleosome C region displayed as a hormone-dependent, MPE-protected region in the wild type and in these two deletion mutants (Figure 5C). Conversely, the nucleosome C region was not protected in the Δ–181/–10 mutant where all GR-binding sites had been deleted. In this mutant, 67% of the wild-type –240/–60 nucleosome B segment has been deleted; however, the –425/–240 nucleosome C region as defined above remains intact. This demonstrates that positioning of nucleosome C depends on the more proximal region of the promoter, which is deleted in the Δ–181/–10 mutant and that contains a strong GR-binding site(s) (Payvar *et al*., 1983).

Therefore, a high affinity GR-binding site(s) seems to be necessary and sufficient for the establishment of

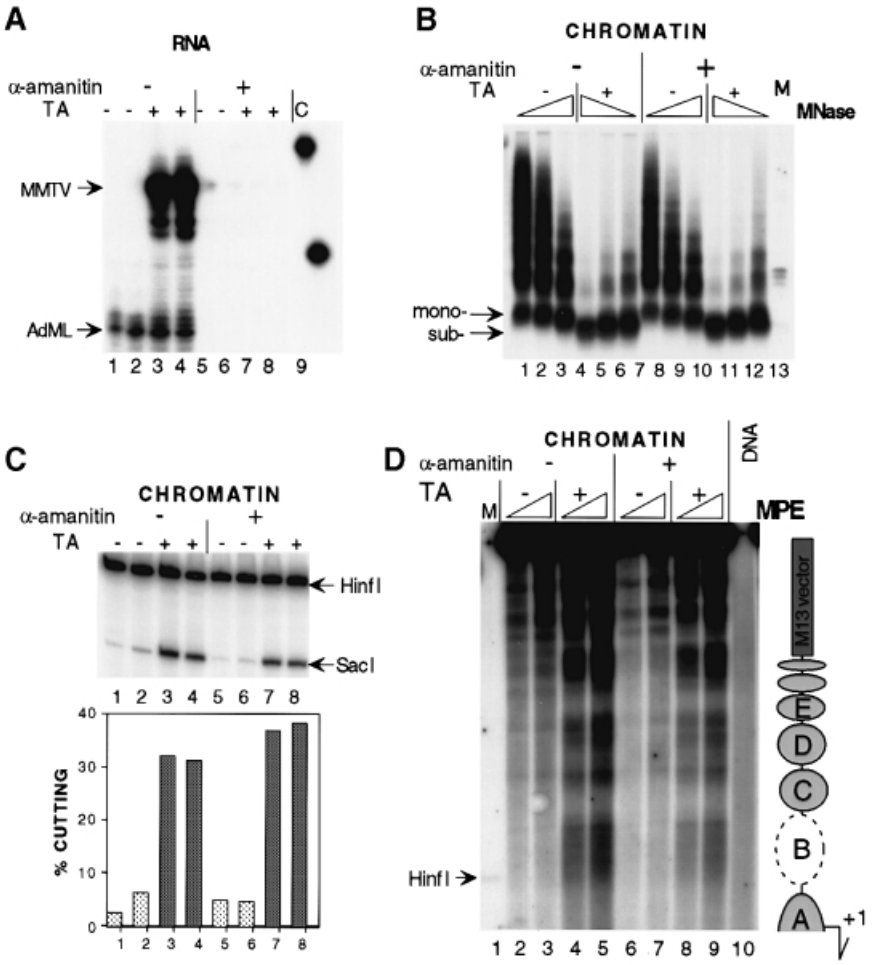

Fig. 6. Nucleosome remodeling and establishment of translational nucleosome positioning are not dependent on ongoing transcription. (**A**) Transcription analysis by S1 nuclease protection of MMTV and AdML RNA. In half of the oocytes, α-amanitin was co-injected together with the DNA (lanes 5–8). After 24 h, 1 μM TA was added (lanes 3, 4, 7 and 8) or not added (lanes 1, 2, 5 and 6) and oocytes were harvested another 24 h later for RNA analysis. Lane 9, undigested S1 probe. (**B**) MNase analysis. DNA was resolved in agarose, transferred and hybridized with a probe encompassing region –192/–100 of MMTV. Arrows show positions for mononucleosomal (mono-) and subnucleosomal (sub-) particles. (**C**) *Sac*I accessibility assay. Oocytes in groups of six for each analysis. Symbols as in Figure 5B. (**D**) MPE footprinting. Oocytes in groups of seven were analyzed by MPE digestion. Isolated DNA was assayed according to the indirect end-labeling protocol as in Figure 4A except that digested DNA was only cleaved with *Eco*RV (+425). Lane 1, internal molecular weight marker, showing the positions of *Hin*fI cleavage. Lane 10, naked dsMMTV promoter DNA digested with MPE. To the right is a schematic summary of MPE cuts along the MMTV LTR with putative nucleosome positions.

chromatin remodeling and nucleosome positioning in the proximal part of the MMTV LTR. Since only 1% of transcriptional activity remained in the Δ–124/–10 mutant, while a significant level of chromatin remodeling and distinct nucleosome positioning was detected, these experiments further suggest that chromatin remodeling can be uncoupled from transcription.

Chromatin remodeling does not depend on ongoing transcription

To achieve a complete transcriptional arrest, we injected α-amanitin, a toxin known for its ability to arrest RNA polymerase II-driven transcription. The toxin was co-injected with ssMMTV DNA and AdML reference DNA. S1 nuclease protection showed that transcription from both the MMTV and the AdML promoter was abolished by α-amanitin at a final intracellular concentration of ~0.5 μg/ml (Figure 6A, lanes 5–8). However, hormone-dependent chromatin remodeling of the MMTV promoter occurred independently of transcription as revealed by (i) MNase (Figure 6B, compare lanes 1–3 and 4–6 with lanes 7–9 and 10–12), (ii) *Sac*I restriction enzyme accessibility assay (Figure 6C, compare lanes 1, 2 and 3, 4 with lanes 5, 6 and 7, 8), and (iii) MPE footprinting (Figure 6D, compare lanes 2, 3 and 4, 5 with lanes 6, 7 and 8, 9). Hormone-activated chromatin remodeling was thus demonstrated to be independent of the transcriptional activity of the MMTV promoter. This was confirmed in a separate experiment in which α-amanitin was injected into the cytoplasm of the oocyte prior to hormone induction (data not shown). Therefore, ongoing transcription is not required for the establishment of chromatin remodeling and nucleosome positioning. This is in agreement with previous results looking at hormone-induced chromatin remodeling of the MMTV promoter in tissue culture cells (Truss *et al*., 1995) and for the thyroid hormone-induced remodeling of the TRβA promoter in *Xenopus* oocytes (Wong *et al*., 1995).

Discussion

The *Xenopus* oocyte has a potential for use as a 'biological test tube' where heterologous DNA and protein can be

highly expressed *in vivo* (Colman, 1984). Studies of thyroid hormone-dependent gene expression have exploited *Xenopus* oocytes to study hormone-induced chromatin remodeling *in vivo* in the TRβA promoter (Wong *et al*., 1998). The optimization of this system by use of ssDNA injection (Almouzni and Wolffe, 1993) and the GR mRNA injection strategy allowed us to demonstrate, for the first time, a hormone-induced nucleosome positioning on the MMTV promoter. In contrast to previous studies of chromatin structure in the MMTV promoter *in vivo* in tissue culture cells (Richard-Foy and Hager, 1987; Fragoso *et al*., 1995; Truss *et al*., 1995) or *in vitro* reconstituted MMTV promoter in *Drosophila* embryo extracts (Venditti *et al*., 1998; Di Croce *et al*., 1999), the *Xenopus* oocytes do not harbor positioned nucleosomes in this promoter prior to hormone activation. This has led to the discovery that GR-mediated chromatin remodeling is able to induce translational nucleosome positioning over this promoter. Furthermore, it shows that a pre-set nucleosome positioning is not required for glucocorticoid-mediated transcription activation but is rather a consequence of the induction event. The nucleosome positioning and additional structural changes in the MMTV promoter depend on the presence of a GRE but are independent of basal promoter elements and of ongoing transcription.

Comparison with previous chromatin studies of MMTV LTR in tissue culture cells

Previous studies of the chromatin structure in the MMTV LTR using tissue culture cells have shown clear-cut hormone-induced effects at the chromatin level. These effects could be monitored as the appearance of a DNase I-hypersensitive site (Zaret and Yamamoto, 1984; Richard-Foy and Hager, 1987; Truss *et al*., 1995), increased restriction enzyme cutting (Archer *et al*., 1992; Truss *et al*., 1995; Fragoso *et al*., 1998) and increased MPE digestion over nucleosome B (Richard-Foy and Hager, 1987). In these studies, nucleosomes were demonstrated to be translationally positioned already in the inactive MMTV LTR (see, however, Fragoso *et al*., 1995), and several careful *in vivo* studies have not detected any effect on nucleosome positioning by hormone activation (Richard-Foy and Hager, 1987; Fragoso *et al*., 1995; Truss *et al*., 1995). The same is true for *in vitro* reconstituted chromatin on subclones of MMTV LTR-derived DNA. Again there is a clear preference in translational positioning of nucleosome B, similar to that found *in vivo*. This is the case both in pure *in vitro* reconstitution systems (Perlmann and Wrange, 1988; Pina *et al*., 1990; Archer *et al*., 1991; Flaus and Richmond, 1998) and in *Drosophila* embryo extracts using plasmid DNA (Venditti *et al*., 1998; Di Croce *et al*., 1999). These *in vitro* studies have demonstrated that MMTV LTR DNA harbors nucleosome positioning elements.

Why is translational nucleosome positioning not observed in the inactive MMTV promoter in *Xenopus* oocytes? Previous *in vivo* studies of MMTV LTR nucleosome positioning in tissue culture cells (Richard-Foy and Hager, 1987; Truss *et al*., 1995) were often based on the use of MMTV reporter constructs propagated in an episomal multicopy BPV vector. Under these conditions, hormone induction engages a minority of the gene copies, 15–20% (Bresnick *et al*., 1992; Fragoso *et al*., 1995). This inevitably results in heterogeneous chromatin patterns. However, studies of the nucleosome structure of the MMTV promoter have also involved cell lines harboring a single copy of stably integrated MMTV LTR-driven reporters (see, for example, Truss *et al*., 1995). These cells also showed a low but significant transcription activity in the absence of exogenously added hormone and again showed no difference in nucleosome positioning in the presence or absence of hormone induction (Truss *et al*., 1995). We speculate that the lack of nucleosome positioning in the *Xenopus* oocytes is due to the complete silence of the MMTV promoter in the absence of added hormone (cf. Figure 1C). A low frequency of transcription may be required and sufficient for nucleosomes to move to their preferred positions along the MMTV LTR, perhaps then retained in these translational positions by DNA sequence-directed positioning elements. This might explain the pre-positioned nucleosomes in MMTV LTR in the tissue culture cell lines. We do not know whether this basal transcription is due to traces of glucocorticoid hormone or to the status of GR or chromatin in these cells. Anyway, this suggests that the *Xenopus* oocyte system offers an unusual opportunity to follow the activation-induced chromatin reorganization of a previously inactive and newly replicated promoter.

Additional differences of possible relevance are the following: in *Xenopus* oocytes, the injected ssDNA is assembled in a process coordinated to the second strand synthesis of our M13 MMTV derivative, presumably without any sequence-specific initiation. In contrast, the BPV vectors used in the previous studies have a defined origin of replication. Furthermore, tissue culture cells are kept at 37°C while *Xenopus* oocytes are kept at 18–19°C. *In vitro* studies show that nucleosome sliding is increased by an increase in temperature (Flaus and Richmond, 1998). Along these lines, we obtained a weak but significant rearrangement of chromatin in the MMTV LTR by incubating the oocytes for 30 min at 37°C prior to homogenization and MPE footprinting (S.Belikov and Ö.Wrange, unpublished observation). In addition, the inactive MMTV LTR in *Xenopus* oocytes does show some weak and variable multiframe nucleosome positioning (see Figure 5C). In none of these cases does the nucleosome pattern become ordered into the strict translational positioning that we obtained in the activated MMTV LTR (Figure 4).

Importantly, our results demonstrate that in the MMTV LTR pre-positioning of nucleosomes is not required to elicit a strong GR-induced response involving chromatin remodeling of virtually all MMTV templates. Rather, the positioning is an integrated part of the chromatin remodeling event. This suggests that GR is able to bind its nucleosomal targets in MMTV LTR irrespective of the translational nucleosome frame in each individual template. This agrees with the results of *in vitro* GR binding (Perlmann and Wrange, 1988) and *in vivo* progesterone receptor binding (Truss *et al*., 1995) studies showing that all five GREs are occupied in spite of their different rotational positioning on the positioned MMTV B nucleosome. Nucleosomes have in several cases been shown to contribute to promoter architecture (Schild *et al*., 1993; Lu *et al*., 1995; Sewack and Hansen, 1997). In these studies, it has been suggested that the main function of a positioned nucleosome is to bring various factor-binding

sites into close proximity. Although our results show that pre-positioning of nucleosomes is not functionally required, they do not exclude the possibility that a stronger and/or a more reactive hormone response may be elicited if nucleosomes are pre-positioned. Such induction kinetics will be difficult to address in *Xenopus* oocytes where lipophilic steroid hormones will be retained for a long time after hormone withdrawal.

Chromatin structure of the activated MMTV promoter

Although hormone activation results in a translationally positioned array of at least six nucleosomes, our results show that each individual nucleosome in this array has a different structure (compare nucleosome B and C in Figures 4A and B, 5C and 6D). Nucleosome B shows hormone-dependent MPE hypercutting, while its upstream neighbor, nucleosome C, shows hormone-dependent protection. These MPE data are consistent with the MNase and DNase I analyses. Nucleosome B is positioned over the GRE, which harbors binding sites for 4–5 GR homodimers (Payvar *et al*., 1983; Truss *et al*., 1995) and includes the NF1 site (Cordingley *et al*., 1987). It is not surprising that the binding of these transactivating factors results in significant remodeling, as revealed by MPE hypercutting, either due to steric effects arising from the factor binding, or due to targeting of chromatin remodeling complex(es) (Fryer and Archer, 1998; Di Croce *et al*., 1999; Rachez *et al*., 1999; Xu *et al*., 1999). The strong and hormone-dependent increase in protection of the neighboring nucleosome C, despite MPE hypercutting in nucleosome B in the active promoter, shows that transactive factor(s)-mediated chromatin disruption is locally restricted. The distinct hormone-dependent remodeling of nucleosome D and to some extent nucleosome E (Figures 4A and B, 5C and 6D) has not been analyzed further but is likely to reflect transactivating factor binding. Several upstream enhancer segments have been found in the MMTV LTR (Gouilleux *et al*., 1991; Lefebvre *et al*., 1991; Kusk *et al*., 1996). We cannot exclude the possibility that the strong upstream MPE-cut sites may represent alternative translational nucleosome frames, but our *in vivo* nucleosome mapping experiments suggest a single frame of nucleosome positioning in the active MMTV promoter.

This study is the first report on the appearance of a subnucleosomal 120 bp particle *in vivo* as well as the hypercutting of MNase in the active MMTV promoter. MNase-induced subnucleosomal particles have been observed previously in other promoters both *in vivo* (Wong *et al*., 1998) and *in vitro* (Liu *et al*., 1999) and represent hallmarks of remodeled promoters during transcription activation. This supports the notion that the subnucleosome observed here does indeed reflect the active structural state of the MMTV promoter. We note that a histone H3–H4 tetramer can form an MNase-protected DNA fragment of 120 bp (Hayes *et al*., 1991) and that partially purified SWI/SNF complex promotes ATP-dependent remodeling of the MMTV B nucleosome octamer DNase I pattern into a tetramer-like pattern *in vitro* (Spangenberg *et al*., 1998). Since the subnucleosome complex is formed following glucocorticoid activation and encompasses a cluster of 4–5 GR-binding sites, it is likely to contain GR. Our efforts are now directed towards the characterization of the protein and DNA components in this subnucleosomal complex.

The mechanism of establishment of hormone-induced translational nucleosome positioning in the MMTV promoter

In vitro nucleosome reconstitution studies suggest that positioning over the nucleosome A and B region in the MMTV LTR is directed to a large extent by the DNA sequence itself (Perlmann and Wrange, 1988; Pina *et al*., 1990; Archer *et al*., 1991; Flaus and Richmond, 1998). We show here, however, that these sequence determinants are overcome in the *in vivo* reconstituted inactive MMTV LTR in *Xenopus* oocytes. On the other hand, during hormone activation, one high affinity GR-binding site is able to mediate nucleosome positioning *in vivo* (Figure 5C). The GR protein has been shown to interact with both the SWI/SNF complex (Yoshinaga *et al*., 1992; Muchardt and Yaniv, 1993; Östlund Farrants *et al*., 1997; Fryer and Archer, 1998) and the histone acetyltransferase-containing coactivator complexes such as GRIP1 (Xu *et al*., 1999). Furthermore, the homologous progesterone receptor was recently shown to target ISWI and NURF38 to the MMTV promoter (Di Croce *et al*., 1999). The ability to reposition nucleosomes along DNA was shown recently for several nucleosome remodeling complexes such as NURF (Hamiche *et al*., 1999), CHRAC (Längst *et al*., 1999) and SWI/SNF complex (Whitehouse *et al*., 1999). For example, in the presence of NURF, nucleosomes in the reconstituted hsp70 promoter adopt one predominant position from a variety of possible locations (Hamiche *et al*., 1999). However, NURF had little effect on 5S nucleosomes, arguing for a definite role of DNA sequence determinants, such as anisotropic flexibility of nucleosomal core DNA and/or rigidity of internucleosomal linker, in establishing nucleosome positioning. It is possible that chromatin remodeling complexes, most probably SWI/SNF (Yoshinaga *et al*., 1992; Östlund Farrants *et al*., 1997; Fryer and Archer, 1998) and/or NURF (Di Croce *et al*., 1999), recruited to the MMTV LTR by GR, participate in chromatin opening. This opening leads to increased nucleosome mobility and allows nucleosomes to slide along the DNA. The sliding then focuses the nucleosomes into a preferred translational frame due to DNA sequence-directed nucleosome positioning and/or sequence-specific DNA-binding transcription factors. These factors may serve as steric barriers, or as nucleosome positioning proteins, as has been shown for HNF3. This factor directs nucleosome positioning in the albumin enhancer (Shim *et al*., 1998). We hypothesize that nucleosome positioning in the MMTV LTR may thus be driven by the cooperative forces of a GR-mediated chromatin remodeling, DNA sequence-directed bending and DNA-binding factors.

Materials and methods

Plasmids, DNA and RNA preparation

The 3 kb *Sal*I–*Pvu*II fragment containing the MMTV LTR/TK gene sequence from pLSwt (Buetti and Kuhnel, 1986) was inserted into M13 mp9 *Sal*I–*Sma* to generate the pMTV:M13 coding strand. The pβGR/RN3P construct designated for *in vitro* transcription of GR RNA was generated by cloning of the *Bam*HI–*Not*I fragment obtained by PCR using specific primers and pBal 117 plasmid (Miesfeld *et al*., 1986)

containing the complete cDNA for rat GR as a template into the vector of *Bam*HI–*Not*I-cleaved plasmid pβGFP/RN3P (Zernika-Goetz *et al*., 1996). 5′-capped GR RNA was prepared by *in vitro* transcription using the Message Machine™ kit (Ambion). Deletion pMTV:M13 coding strand mutants were created by 'long' PCR using primers containing a unique *Xho*I recognition site and positioned such that the indicated DNA segments were deleted in the final constructs (see Figure 5A) with subsequent cleavage with *Xho*I and ligation. The success of cloning was confirmed by sequencing.

Oocyte microinjection and maintenance

Defolliculated stage VI *Xenopus laevis* oocytes were prepared by collagenase treatment (Almouzni and Wolffe, 1993). The oocytes were incubated overnight at 18–19°C in OR2 medium containing 1 mM $CaCl_2$ (Colman, 1984). The next day, healthy oocytes were injected with DNA into nuclei and/or RNA into the cytoplasm (20 nl). In a typical experiment, 10 ng of *in vitro* transcribed GR RNA were injected and 2–6 h later 1 ng of ssDNA of pMTV:M13 coding strand and 0.25 ng of pAdML dsDNA (Ohlsson and Edlund, 1986) were injected, the latter as a transcription reference (Perlmann and Wrange, 1991). In the experiments indicated, GR was introduced as a GR expression vector (pSTC GR 3-795) (Wieland *et al*., 1988), which was co-injected with the other DNAs as 5 ng of dsDNA. At 16–24 h after injections, the synthetic glucocorticoid hormone TA (Sigma-Aldrich), routinely at 1 μM, was added to the oocyte culture medium. Oocytes were harvested for analysis at the time indicated. For RNA polymerase II transcription inhibition, 20 nl of α-amanitin was co-injected into oocyte nuclei at a concentration of 30 μg/ml in the course of the DNA injection.

RNA analysis and immunoblotting

RNA analysis and immunoblotting using a polyclonal rabbit antiserum against rat GR were performed as previously described (Gelius *et al*., 1999).

DNase I hypersensitivity assay

Twelve injected oocytes were collected and homogenized in 10 mM Tris–HCl pH 8.0, 50 mM NaCl, 1 mM dithiothreitol (DTT), 5 mM $MgCl_2$ and 5% glycerol (buffer A) (three oocytes in 100 μl) by pipeting up and down at 0°C until an even homogenate is obtained (~15–20 times). The homogenate was divided into four tubes and DNase I (Boehringer Mannheim) was added (6, 9, 13.5 and 20.25 U per tube). Following incubation at 20°C for 3 min, the reaction was stopped by addition of SDS to 0.5% and EDTA to 10 mM. DNA was purified by proteinase K treatment, phenol/chloroform extractions and ethanol precipitations. The samples were RNase A treated (30 μg/ml) for 1 h at 37°C and purified DNA cleaved with *Sal*I, resolved in a 1% agarose gel (Sambrook *et al*., 1989), vacuum transferred and hybridized with the ^{32}P-labeled single-stranded *Sal*I–*Sac*I fragment of pLSwt (Buetti and Kuhnel, 1986), obtained by synthesis of cDNA (Church and Gilbert, 1984).

MNase digestion

Injected oocytes in groups of 10 were homogenized in buffer A with 3 mM $CaCl_2$ (three oocytes in 100 μl). The homogenate was divided into three parts and MNase (Amersham Pharmacia Biotech) was added (15, 30 and 60 U per tube). After 5 min incubation (20°C), the reaction mixture was adjusted to 0.5% with respect to SDS and 10 mM EDTA. DNA was purified as above, resolved in a 1.6% agarose gel in 0.5× TBE (Sambrook *et al*., 1989), blotted and hybridized in succession with different random-primed ^{32}P-labeled probes as indicated.

SacI in situ accessibility assay

Oocytes in groups of 5–10 were rinsed twice in *Sac*I *in situ* buffer [20 mM Tris–HCl pH 8.3, 40 mM NaCl, 1 mM $MgCl_2$, 0.5 mM spermine, 0.15 mM spermidine, 5% glycerol (v/v), 1 mM DTT] and then homogenized in 10 μl per oocyte of the same buffer. We found that the pH of the oocyte homogenization buffer used in the *Sac*I *in situ* cutting experiments had a surprisingly dramatic effect selectively on the hormone-dependent *Sac*I cutting: the hormone-dependent stimulation of *Sac*I *in situ* cutting increased from nearly undetectable levels at pH 7.0–7.4 to 5- to 10-fold at pH 8.0–8.6 (data not shown); *Sac*I cutting of naked MMTV DNA in *Xenopus* oocyte homogenates did not display this profile.

Eight units of *Sac*I restriction endonuclease (NE Biolabs) were added per oocyte and incubated for 10 min at 18°C. The reaction was stopped by addition of SDS, EDTA and proteinase K to a final concentration of 1%, 30 mM and 325 μg/ml, respectively. DNA was then extracted as above. The purified DNA was cleaved to completion by 2 U of *Hin*fI and 50 μg/ml RNase A in 100 μl of 1× *Hin*fI buffer. The reaction was stopped by adding an equal volume of phenol/chloroform (2:1), and DNA was purified as above, dissolved in 10 μl of 10 mM Tris–HCl pH 7.5 and 0.1 mM EDTA. One half to one oocyte equivalent was then analyzed by primer extension using a ^{32}P-labeled primer (–291/–265, coding strand), which was labeled by use of T4 polynucleotide kinase (Sambrook *et al*., 1989). Primer extension generated a 183 and a 221 nucleotide product when cleaved at *Sac*I or *Hin*fI, respectively (Figure 1A). Primer extension reaction was carried out in a 10 μl volume containing the sample DNA, 0.5 pmol of 5′-end-labeled primer, 0.2 mM each of the four dNTPs and 0.2 μl (0.4 U) of Vent polymerase exo™ (NE Biolabs) in 1× Vent polymerase buffer. Primer extension was performed in a thermal cycler under standard conditions (95°C/1 min; 55°C/2 min; 72°C/3 min) for 20 cycles followed by analysis on a 6% polyacrylamide sequencing gel. '*Sac*I cutting' was always calculated as *Sac*I-cut/total DNA (i.e. the sum of *Hin*fI- and *Sac*I-cut DNA) as quantified by PhosphorImager analysis (Molecular Dynamics)

In situ cleavage by MPE

Seven injected oocytes were homogenized in 350 μl of MPE digestion buffer (10 mM Tris–HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 0.15 mM spermidine, 0.5 mM spermine, 0.3 M sucrose) and hydrogen peroxide was added to oocyte homogenate at a final concentration of 1–2 mM. MPE·iron(II) (Sigma-Aldrich) complex was prepared by mixing 18.8 μl of 625 μM MPE solution (from the 5 mM aqueous stock) with 18.8 μl of 625 μM ferrous ammonium sulfate solution (from freshly prepared 5 mM aqueous stock). Immediately before use, DTT was added to the solution of MPE·Fe(II) to achieve a final concentration of 10 mM (from a freshly prepared 1 M stock). The cleavage reaction was started by mixing oocyte homogenate with MPE·Fe(II) complex and incubation was at 25°C for the indicated time. The reaction was stopped by addition of 1/10 volume of 50 mM bathophenanthroline disulfonate (Merck). DNA was isolated as described above and cleaved by restriction enzyme for indirect end-labeling assay (Wu, 1989).

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