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Inhibition of Chromatin Assembly in *Xenopus* Oocytes Correlates with Derepression of the Mouse Mammary Tumor Virus Promoter

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The mouse mammary tumor virus (MMTV) promoter is positively regulated by glucocorticoid hormone via binding of glucocorticoid receptor to a specific response element. Upon addition of hormone, a nucleosome containing the glucocorticoid response element is removed or structurally altered, suggesting that the nucleosome interferes with transcription. Accordingly, inhibition of chromatin assembly should relieve the repression and result in an increased constitutive activity. We have tested this hypothesis by injecting nonspecific competitor DNA into *Xenopus laevis* oocytes to titrate endogenous histones. The coinjection of competitor DNA altered chromatin structure: nucleosomal ladders produced by micrococcal nuclease were disrupted, and the unique helical setting of the MMTV promoter in a nucleosome was lost, as shown by in situ DNase I footprinting. Basal MMTV transcription was drastically increased by competitor DNA, whereas a coinjected, constitutively active adenovirus 2 major late promoter was not stimulated. These results show that the uninduced MMTV promoter is under negative control and provide direct support for the theory that the nucleosomal organization maintains the repression of this promoter in its uninduced state.

The glucocorticoid receptor (GR) is a ligand-activated transcription factor belonging to a large family of soluble intracellular hormone receptors including the steroid, thyroid, retinoic acid, and vitamin D receptors (for a review, see reference 10). GR activates some promoters and represses others (9). Transcriptional activation involves binding of the receptor to specific DNA sequences termed glucocorticoid response elements. In the glucocorticoid-activated promoter of the mouse mammary tumor virus long terminal repeat (MMTV LTR), the glucocorticoid response element is composed of several binding sites for GR, in addition to a binding site for nuclear factor 1 (NF-1) (4, 22). These sequences have been shown to coincide with a DNase I-hypersensitive region in the chromatin of hormone-treated cells (36). Richard-Foy and Hager (26) demonstrated that nucleosomes are specifically positioned in the MMTV LTR present in episomal bovine papillomavirus vectors. A nucleosome positioned over the glucocorticoid response element and the binding site for NF-1 were altered upon hormone treatment of the cells. By an in situ exonuclease III protection assay, Cordingley et al. (8) showed that NF-1 binding to the regulatory region was hormone dependent. Taken together, these results suggest that the uninduced MMTV promoter is transcriptionally repressed by a well-defined chromatin structure and that GR induction involves a local change of the chromatin conformation, which leads to binding of NF-1 and other transcription factors.

Several other promoters show correlation of activity and regions of altered chromatin, indicating that the appearance of nucleosome-free regions is involved, as either a cause or a consequence of activity (1, 20, 32). Support for a direct involvement of nucleosomes in transcriptional regulation comes from studies of yeast cells, in which the only functional histone H4 gene was placed under the control of an inducible promoter, allowing the cells to become histone H4 depleted (12, 13). Furthermore, several investigators have shown that nucleosomes inhibit transcription initiation in vitro. This inhibition can be relieved by prebinding of the general transcription factor TFIID, as well as upstream activators, before nucleosomes are assembled on the template, again indicating a negative regulatory role for nucleosomes (34, 35).

We have investigated the functional importance of the nucleosomal organization of the MMTV promoter in *Xenopus laevis* oocytes. The promoter can be strongly induced by glucocorticoids in these cells if an expression vector encoding GR is also present. Since nucleosomes are formed on plasmids injected into oocyte nuclei, we reasoned that the regulatory role for nucleosomes in the MMTV promoter could be tested if nucleosome assembly could be inhibited on injected MMTV plasmids. We have achieved this by coinjecting competitor DNA along with promoter plasmids, thereby blocking the formation of nucleosomes. This resulted in strongly increased basal transcription from the MMTV promoter, while a constitutively active adenovirus 2 major late (AdML) promoter was unaffected. Our results provide direct evidence for negative control of the MMTV promoter in the absence of hormone and suggest that this repression is caused by the nucleosomal organization of the promoter.

MATERIALS AND METHODS

Plasmids and competitor DNA. Plasmids used for oocyte injection were pBalE1 (21), containing the AdML promoter; HGI, encoding the human glucocorticoid receptor under the control of the simian virus 40 (SV40) early promoter and enhancer region (17); and pLSwt containing the MMTV LTR and pLS-181/-169 (4), carrying a *Hin*dIII linker at positions −181 to −169, designated MMTV mut. in Fig. 1. pGEM-1 and/or poly(dI-dC) · poly(dI-dC) (Pharmacia) were used as competitor DNAs in the experiments whose results are shown in Fig. 2, 3, 4 and 5. The ^{32}P-labelled plasmids used in

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the experiments in Fig. 2 and 3 were pLSwt and pGEM-1, respectively, which had been opened with *Eco*RI, treated with calf intestinal alkaline phosphatase, 5′ end labelled with [γ-^{32}P]ATP and polynucleotide kinase, and finally recircularized with T4 DNA ligase (19).

Oocyte injections. Animals and oocytes were handled as described previously (7). Plasmids were dissolved in H_2O in a total volume of 20 to 30 nl and injected into the nuclei of collagenase (type II; Sigma)-treated stage VI *Xenopus laevis* oocytes. Oocytes were incubated in modified Bart's solution (7) for 24 to 48 h at 20°C. Where indicated, 10^{-7} M glucocorticoid hormone (triamcinolone acetonide) was included in the incubation medium. In the experiments whose results are shown in Fig. 2 and 4, nuclei from injected oocytes were isolated by dissection and gently homogenized in H buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 25% glycerol, 12.5% sucrose, 1 mM dithithreitol, 0.5 mM phenylmethylsulfonyl fluoride).

RNA analysis. Injected oocytes were pooled in groups of 20 to 30, and total RNA was recovered as previously described (7). RNA from three oocyte equivalents was hybridized with a mixture of probes derived by cleavage of pBalE1 with *Sst*I and *Hin*dIII (−405 to +192 in relation to the transcription start site) and of pLSwt with *Sst*I and *Bam*HI (−104 to +134). Prior to mixing with RNA, the probes were end labelled by treatment with polynucleotide kinase and [γ-^{32}P]ATP. After overnight hybridization at 46°C in 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4)–400 mM NaCl–1 mM EDTA in 80% formamide, samples were treated with 150 U of S1 nuclease (Pharmacia) in S1 nuclease buffer (250 mM NaCl, 30 mM sodium acetate [pH 4.8], 1 mM $ZnCl_2$) and run on 6% polyacrylamide sequencing gels; ^{32}P-labelled markers were included on the gels to determine the positions for correctly initiated transcripts.

Micrococcal nuclease treatment. Labelled and recircularized (see above) pLSwt (2×10^3 cpm per oocyte; less than 1 ng) was coinjected with or without 100 ng of poly(dI-dC) · poly(dI-dC) (Pharmacia). Nuclei were isolated 20 to 24 h after injection, gently homogenized in H buffer (25 μl per nucleus), treated with 12 U of micrococcal nuclease (Boehringer) per 50-μl reaction mixture, and incubated at ambient temperature as indicated in the legend to Fig. 2. Total nucleic acid was recovered, and 5,000 cpm was applied per lane and run on 2% agarose gels in Tris-acetate electrophoresis buffer (19); the gels were subsequently dried and exposed on X-ray film.

Analysis of nucleosome density. Indicator plasmid (pGEM-1) was labelled as pLSwt for the experiment whose results are shown in Fig. 2 (see above). Plasmid corresponding to 2×10^3 cpm (less than 1 ng) was injected into each oocyte, together with 0, 100, or 200 ng of either pGEM-1 vector or synthetic poly(dI-dC) · poly(dI-dC) (Pharmacia). Oocytes were incubated in modified Bart's solution at 20°C for 16 h and pooled in groups of 10 to 20 oocytes. Total nucleic acid was recovered, and 5,000 cpm was run per lane on 1% agarose gels in Tris-acetate electrophoresis buffer containing 0, 0.2, 0.5, and 0.8 μg of chloroquine (Sigma) per ml in the gels in Fig. 3A, B, C, and D, respectively. The gels were dried and exposed on X-ray films. In Fig. 5, 5,000 cpm was recovered from oocytes injected with labelled DNA as described above and run on 1% agarose gels containing 0.5 μg of chloroquine per ml.

In situ DNase I footprinting. Oocytes were injected with 10 ng of pLSwt, either with or without coinjected poly(dI-dC) · poly(dI-dC), and incubated in modified Bart's solution at 20°C for 16 h. Nuclei were dissected and gently homogenized in H buffer (25 μl per nucleus); incubation (0.5 oocyte equivalents per reaction) was for 30 s with 5.6 μg of DNase I (grade I; Boehringer) per ml at ambient temperature. Samples were treated with sodium dodecyl sulfate and proteinase K; DNA was recovered by ethanol precipitation and then subjected to 25 cycles of amplification with AmpliTaq polymerase (Perkin-Elmer Cetus) in a Perkin-Elmer Cetus DNA thermal cycler, as described previously (28). The primers consisted of sequences from the MMTV LTR: from −291 to −265 in the upper strand and from +10 to +30 in the bottom strand. Three hundred fifty femtomoles of either primer, labelled by treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP, was added to each amplification reaction mixture. DNA was recovered and run on 6% polyacrylamide denaturing sequencing gels.

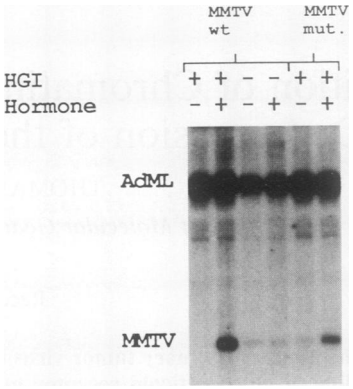

FIG. 1. S1 analysis of RNA derived from injected oocytes. All oocytes were injected with the AdML promoter plasmid (0.1 ng per oocyte) as a reference. HGI, expressing the gene for the human glucocorticoid receptor, was either injected (10 ng per oocyte) (+) or not injected (−). PLSwt (5 ng per oocyte), here designated MMTV wt and containing the wild-type MMTV promoter, was used as reporter promoter in the first four lanes, while a mutant MMTV promoter (MMTV mut.) (5 ng per oocyte) was used in the last two lanes. Oocytes were incubated for 48 h in the presence or absence of 10^{-7} M hormone (triamcinolone acetonide) as indicated (+ or −). AdML and MMTV indicate the positions in the gel where correctly initiated transcripts migrated, protected by the AdML probe and the MMTV probe, respectively.

RESULTS

Glucocorticoid-dependent transcription. We first tested whether the MMTV promoter is regulated by hormone after introduction into *Xenopus* oocytes. As demonstrated in Fig. 1, hormone-dependent transcription from the MMTV promoter can be established in oocytes. In these experiments, three different plasmids were coinjected into the nuclei of the cells: a vector expressing the human glucocorticoid receptor gene (HGI) (17), a reporter carrying the glucocorticoid-regulated MMTV promoter (MMTV wt), and a reference providing the constitutively active AdML promoter. Correctly initiated transcripts were detected by S1 nuclease protection analysis (Fig. 1). MMTV transcription was strongly stimulated by the addition of hormone (more than 300-fold in the experiment whose results are shown in Fig. 1). The low basal level and the strong hormonal stimulation are in agreement with the behavior of the MMTV promoter in cultured mammalian cells (4, 17, 26). Hormone-induced MMTV transcription was dependent on coinjection of GR

expression vector (Fig. 1, HGI), showing that oocytes are deficient in endogenous GR activity. A mutant glucocorticoid response element, previously shown to eliminate GR binding to one of two binding regions in the promoter (23), reduced the hormone-induced level of transcription in oocytes (Fig. 1, MMTV mut.) to approximately the same extent as observed in cultured cells with this mutant construct (4). Thus, activation was mediated by GR binding to the promoter. We conclude that GR is the only additional requirement for glucocorticoid-regulated activation of the MMTV promoter in these cells. Similar results, describing estrogen-induced expression from the vitellogenin B1 promoter in *Xenopus* oocytes, have been reported previously (31).

Inhibition of chromatin assembly. Nucleosomes have previously been shown to form on DNA injected into nuclei of *Xenopus* oocytes. For example, if injected oocytes are gently homogenized and treated with micrococcal nuclease, the digested DNA forms regular ladders after gel electrophoresis (11). Individual bands are separated by approximately 180 bp, indicating that regularly spaced nucleosomes have assembled on the injected DNA.

The MMTV promoter has been proposed to be negatively controlled by a specifically positioned nucleosome (see above). To provide support for this model, we wished to inhibit the formation of nucleosomes on injected plasmids.

Assembly was blocked by coinjecting competitor DNA [pGEM-1 (Promega) or poly(dI-dC) · poly(dI-dC) (Pharmacia)] together with promoter plasmids into oocytes in order to titrate endogenous histones. To study the chromatin formed on injected DNA, plasmids were ^{32}P labelled (see Materials and Methods) and injected either alone or together with competitor DNA. After 12 to 15 h of incubation, nuclei were dissected, homogenized, and treated with micrococcal nuclease for various time periods. Samples were deproteinized and analyzed on agarose gels. A resulting autoradiogram is shown in Fig. 2. Micrococcal nuclease-digested samples from oocytes injected with ^{32}P-labelled DNA alone (lanes 5 to 8) displayed a regular ladder, with approximately 180 bp between individual bands, indicating that normally spaced nucleosomes were formed on the injected DNA. After prolonged digestion, a band corresponding mainly to mononucleosomes (at approximately 140 bp) was detected (lanes 7 and 8). Labelled DNA injected together with 100 ng of poly(dI-dC) · poly(dI-dC) competitor gave much weaker and shorter nucleosomal ladders (Fig. 2, compare lanes 1 and 5). In addition, significant amounts of plasmid DNA always migrated ahead of the mononucleosome band, indicating that a large DNA fraction lacked nucleosomes in the presence of competitor DNA (Fig. 2, lanes 1 to 4). This experiment showed that 100 ng of poly(dI-dC) · poly(dI-dC) (Pharmacia) is sufficient for drastic reduction of nucleosome formation on coinjected plasmids. Identical results are seen with pGEM-1 as coinjected competitor (data not shown).

Negative superhelicity is introduced in the injected plasmids as a consequence of the nucleosome organization. The superhelicity therefore reflects the density of nucleosomes assembled on the injected DNA (11) and can be monitored by agarose gel electrophoresis. Accordingly, such gels showed that the covalently closed, recircularized ^{32}P-labelled plasmids became negatively supercoiled after injection into oocytes (Fig. 3A, form I, lane 2). Injection of 100 or 200 ng of nonspecific vector DNA resulted in a drastic decrease in nucleosome density on the coinjected ^{32}P-labelled plasmid, as evident from the lower degree of superhelical density (Fig. 3A, compare form I in lanes 3 and 4,

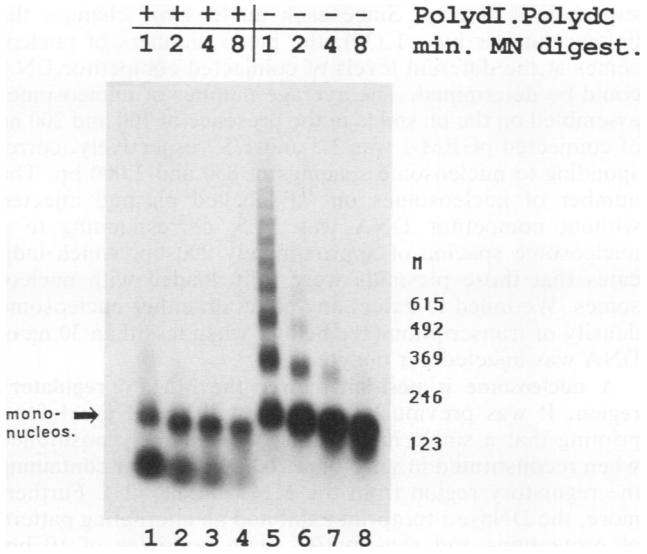

FIG. 2. Micrococcal nuclease treatment of oocyte nuclei. Nuclei were isolated from injected oocytes and treated with nuclease for 1, 2, 4, and 8 min at ambient temperature. Molecular weight markers as well as the positions corresponding to mononucleosomes are indicated. Lanes 1 to 4, autoradiogram from an agarose gel with samples from oocytes injected with labelled pLSwt together with 100 ng of poly(dI-dC) · poly(dI-dC). Lanes 5 to 8, autoradiogram as above, but with samples from nuclei injected with labelled pLSwt in the absence of competitor. min. MN digest., minutes of micrococcal nuclease digestion. mononucleos., mononucleosomes.

respectively). In ethidium bromide-stained gels, we have observed that injected negatively supercoiled plasmid was relaxed when 200 ng of pGEM-1 was injected, showing that the decrease in negative superhelicity in the labelled plasmids shown in Fig. 3 was not due to inhibition of topoisomerase activity by coinjected nonspecific DNA (data not shown). The decrease in negative superhelicity can be quantitated on agarose gels with various amounts of DNA intercalator (e.g., chloroquine) (Fig. 3B to D) (16). In these gels, plasmid DNA became more positively supercoiled because of intercalating chloroquine; several bands were seen in most lanes, since the covalently closed plasmids were composed of a distribution of topoisomers. Therefore, also the topoisomers in the supercoiled DNA (Fig. 3A, lanes 2 and 3) were resolved, which allowed the determination of

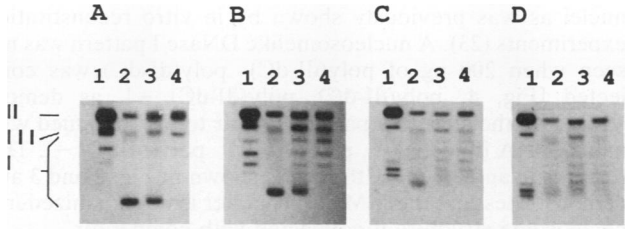

FIG. 3. Nucleosome density. Autoradiograms from 1% agarose gels containing 0, 0.2, 0.5, and 0.8 μg of chloroquine per ml in panels A, B, C, and D, respectively. Approximate positions for form I (covalently closed), form II (nicked circular), and form III (linear) plasmid DNA are indicated. Uninjected labelled indicator plasmid (pGEM-1) was applied in lanes 1. Samples from oocytes injected with 0, 100, and 200 ng of pGEM-1 vector DNA as nonspecific competitors were applied in lanes 2, 3, and 4, respectively.

superhelical density. Since each nucleosome changes the linking number by −1 (30), the mean numbers of nucleosomes at the different levels of coinjected competitor DNA could be determined. The average number of nucleosomes assembled on the plasmids in the presence of 100 and 200 ng of coinjected pGEM-1 was 3.5 and 1.5, respectively, corresponding to nucleosome spacings of 850 and 2,000 bp. The number of nucleosomes on ^{32}P-labelled plasmid injected without competitor DNA was 14.5, corresponding to a nucleosome spacing of approximately 200 bp, which indicates that those plasmids were fully loaded with nucleosomes. We failed to detect any effect on either nucleosome density or transcription (see below) when less than 30 ng of DNA was injected per oocyte.

A nucleosome is positioned over the MMTV regulatory region. It was previously demonstrated by DNase I footprinting that a single nucleosome was uniquely positioned when reconstituted in vitro on a 200-bp fragment containing the regulatory region from the MMTV LTR (23). Furthermore, the DNase I footprint exhibited an alternating pattern of protections and sensitivities, with multiples of 10 bp, indicating that the nucleosome had a strictly defined helical setting (23). To determine whether a nucleosome was similarly organized in injected MMTV DNA, nuclei from injected oocytes were dissected, homogenized, and treated with DNase I. The digested DNA was subsequently amplified by multiple primer extensions with *Taq* polymerase (28). Figure 4 shows the DNase I ladders originating from the coding and noncoding strands of the MMTV promoter. The pattern obtained from injected MMTV plasmid alone [injected +, poly(dI-dC) · poly(dI-dC) −] was altered compared with the pattern from uninjected plasmid [injected −, poly(dI-dC) · poly(dI-dC) −], indicating that the injected DNA was complexed with proteins. The alterations extend over at least 130 bp, and there was a tendency for a pattern of multiples of 10 bp alternating protections and sensitivities; see, e.g., the sensitive positions −96, −108, −127, and −138 in the noncoding strand with protected regions in between. The positions which were sensitive to DNase I attack in the in vitro reconstituted MMTV nucleosome (23) are indicated in Fig. 4B for comparison (asterisks); the pattern is clearly similar to that obtained from the MMTV plasmids injected into the oocyte without competitor DNA (open arrows). This is particularly evident in the noncoding strand, in which most of the DNase I-sensitive sites are located in similar positions. In the coding strand, the tendency is not as obvious but nevertheless shows clear similarities. See, e.g., positions −196, −166, −157, −114, −104, and −94. These results suggest that a nucleosome was organized with a similar helical setting in the MMTV promoter in oocyte nuclei as was previously shown by in vitro reconstitution experiments (23). A nucleosomelike DNase I pattern was not seen when 200 ng of poly(dI-dC) · poly(dI-dC) was coinjected [Fig. 4, poly(dI-dC) · poly(dI-dC) +], as demonstrated by the shift to a pattern similar to that obtained with naked DNA [injected −, poly(dI-dC) · poly(dI-dC) −]. This result is in agreement with results shown in Fig. 2 and 3 and demonstrates that the MMTV promoter is not organized in a nucleosome structure if coinjected with competitor.

Inhibition of chromatin assembly increases basal transcription. Basal, uninduced transcription from the MMTV promoter was evaluated in the presence and absence of competitor DNA. The injected oocytes were incubated overnight, and specific transcripts were then quantitated by S1 protection analysis. Oocytes which had been coinjected with 100 ng of vector DNA showed a strongly stimulated basal MMTV transcription (Fig. 5B), whereas transcription from the reference plasmid (AdML) was slightly reduced. Two hundred nanograms of injected competitor DNA also stimulated the MMTV promoter, but to a lesser extent, and further reduced transcription from the AdML promoter. As for Fig. 3, the degree of superhelicity was monitored in parallel injection experiments by using ^{32}P-labelled plasmids and competitor DNA (Fig. 5A and C). The synthetic double-stranded polynucleotide poly(dI-dC) · poly(dI-dC) (Pharmacia) inhibited formation of nucleosomes (Fig. 5C) and caused derepression of the MMTV promoter to a similar extent as pGEM-1 (Fig. 5D).

The experiments showed that vector DNA, as well as poly(dI-dC) · poly(dI-dC), could relieve a negative control from the uninduced MMTV promoter and thereby cause hormone- and GR-independent promoter activation. The drastically increased transcription was not an effect of competition with factors exerting a negative effect on all promoters, since the AdML promoter was not stimulated. In contrast, the decreasing transcription from the AdML promoter, in the presence of increasing amounts of injected competitor DNA, probably reflected titration of positive transcription factors; this may also explain why the activity of the MMTV promoter was not increased further when 200 ng of competitor DNA was injected.

DISCUSSION

We have described experiments that provide evidence that supports the hypothesis that nucleosome structure maintains the low basal level of the MMTV promoter.

A role for nucleosomes as general repressors of transcription has previously been demonstrated in a number of in vitro studies. For example, Lorch et al. (18) showed that transcription was blocked by a nucleosome reconstituted onto a promoter-containing fragment if the initiation site was included in the nucleosome. In other studies using plasmid DNA and *Xenopus* extract to reconstitute chromatin, transcriptional repression caused by the assembly of nucleosomes could be prevented by prebinding of certain transcription factors (34, 35).

In vivo experiments performed with a yeast strain, in which the only functional histone H4 gene was on a plasmid under the control of the inducible GAL promoter, have clearly shown that intact chromatin structure plays an important role in maintaining a low basal level of transcription from various promoters (12, 13). In these experiments, alterations of an intact chromatin structure resulted in transcription from several inducible promoters, whereas others (constitutively active) were unaffected. Furthermore, these experiments demonstrated that the level of induction reached by histone H4 depletion was, in many cases, not influenced by the presence or absence of upstream activating sequences, indicating that some yeast enhancer elements may have a role in opening up chromatin structure.

The chromatin is highly organized in the MMTV promoter present on bovine papillomavirus minichromosome vectors in tissue culture cells (26). This seems to be the case also when the MMTV region is introduced in *Xenopus* oocyte nuclei, since we see evidence for specific helical setting of the MMTV promoter DNA. This conclusion comes from high-resolution in situ DNase I footprints that resulted in a tendency for a 10-bp repeated pattern in the MMTV plasmids injected without competitor [Fig. 4, poly(dI-dC) · poly(dI-dC) −] and is typical for DNA uniquely positioned on the surface of a nucleosome core. Interestingly, the

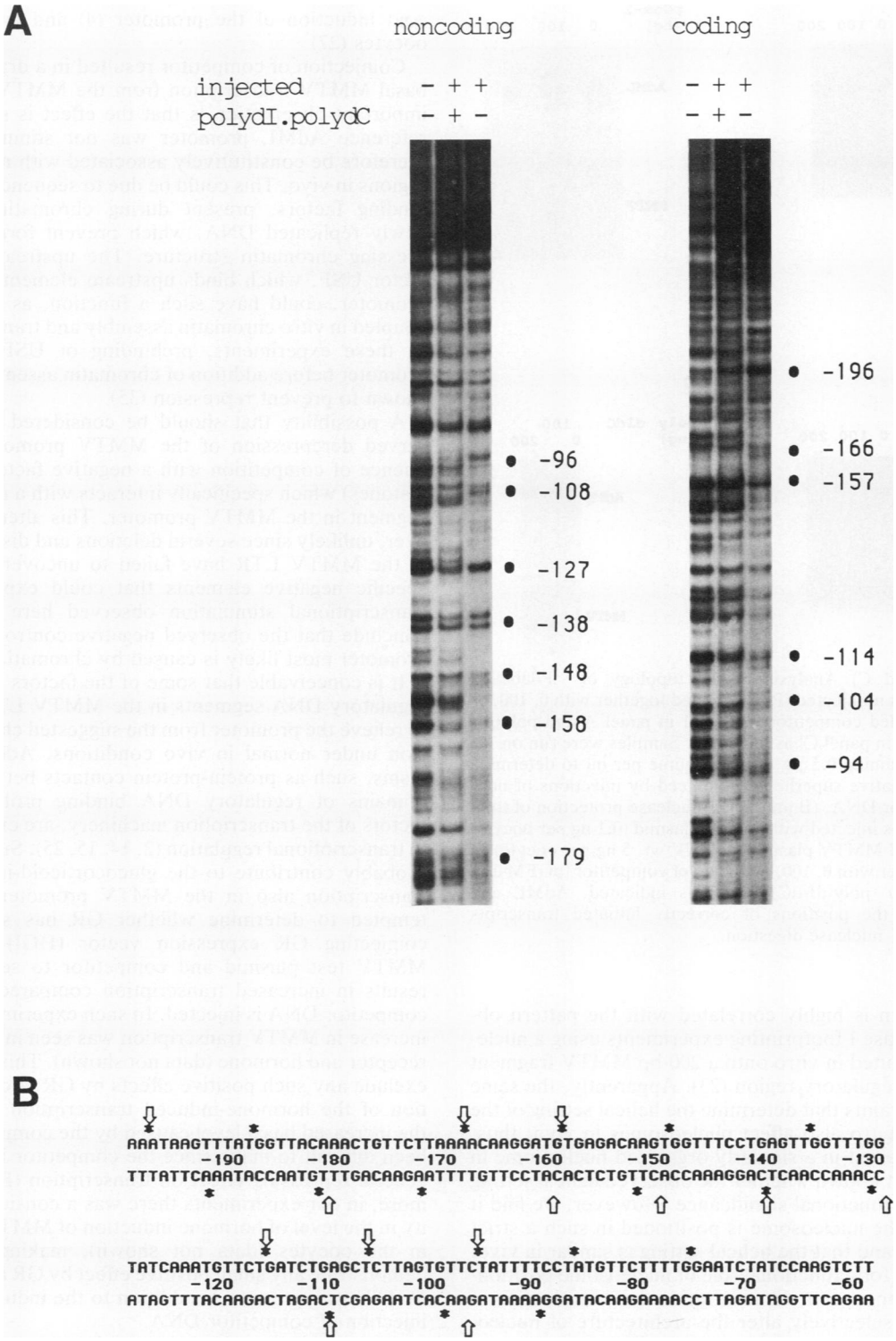

FIG. 4. (A) In situ DNase I footprinting of MMTV wt. MMTV wt was injected into oocytes with (+) or without (−) poly(dI-dC) · poly(dI-dC). Injected oocytes were incubated for 15 h at 20°C. In the control lane, uninjected MMTV wt (injected, −) was treated in a manner similar to the DNA injected into oocytes (i.e., DNase I treated and amplified). Dots indicate increased DNase I-sensitive positions in the MMTV region injected without competitor. (B) MMTV sequence from −58 to −198 upstream of the transcription start site. Arrows indicate the DNase I-sensitive positions in situ established from the above footprint experiment. Asterisks indicate DNase I-sensitive positions in the in vitro reconstituted nucleosomes previously established (23) (see text for details).

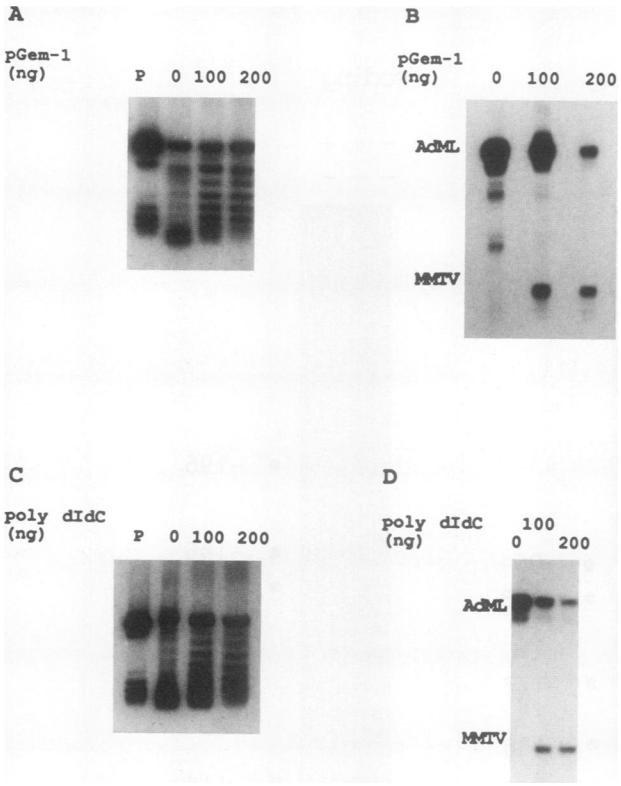

FIG. 5. (A and C) Analysis of the topology of ^{32}P-labelled indicator plasmids uninjected (P) or injected together with 0, 100, or 200 ng of unlabelled competitor [pGEM-1 in panel A and poly(dI-dC) · poly(dI-dC) in panel C] as indicated. Samples were run on 1% agarose gels containing 0.5 μg of chloroquine per ml to determine differences in negative superhelicity induced by injections of non-specific competitor DNA. (B and D) S1 nuclease protection of total RNA from oocytes injected with AdML plasmid (0.1 ng per oocyte) as a reference and MMTV plasmid (MMTV wt, 5 ng per oocyte) as a reporter, together with 0, 100, or 200 ng of competitor [pGEM-1 in B or poly(dI-dC) · poly(dI-dC) in D] as indicated. AdML and MMTV indicate the positions of correctly initiated transcripts protected from S1 nuclease digestion.

observed pattern is highly correlated with the pattern obtained from DNase I footprinting experiments using a nucleosome reconstituted in vitro onto a 200-bp MMTV fragment containing the regulatory region (23). Apparently, the same sequence constraints that determine the helical setting of the nucleosome in vitro also affect nucleosomes in vivo; thus, the region is present in a similarly organized nucleosome in oocytes. It is not known whether the observed helical setting per se is of any functional significance. However, we find it intriguing that the nucleosome is positioned in such a strict manner in vitro and that the helical setting is similar in vivo. Direct evidence for a functional role of nucleosome positioning comes from the recent demonstration that introduction of sequences that selectively alter the architecture of nucleosomes around a yeast autonomously replicating sequence affected the ability of these sequences to support replication (29).

Because of the high copy number of injected MMTV plasmids, the transcription seen in Fig. 3D (when 200 ng of competitor was injected) is probably restricted to only a minority of the MMTV copies. This would explain why we were unable to detect any NF-1 footprints in the MMTV promoter coinjected with 200 ng of competitor, although this transcription factor has a positive effect on the glucocorticoid induction of the promoter (4) and is present in the oocytes (27).

Coinjection of competitor resulted in a drastic increase in basal MMTV transcription from the MMTV promoter. An important observation is that the effect is not general; the reference AdML promoter was not stimulated and may therefore be constitutively associated with nucleosome-free regions in vivo. This could be due to sequence-specific DNA binding factors, present during chromatin assembly on newly replicated DNA, which prevent formation of a repressing chromatin structure. The upstream transcription factor USF, which binds upstream elements in the AdML promoter, could have such a function, as suggested by a coupled in vitro chromatin assembly and transcription assay. In these experiments, prebinding of USF to an AdML promoter before addition of chromatin assembly extract was shown to prevent repression (35).

A possibility that should be considered is that the observed derepression of the MMTV promoter is a consequence of competition with a negative factor (unrelated to histones) which specifically interacts with a regulatory DNA segment in the MMTV promoter. This alternative is, however, unlikely since several deletions and discrete mutations in the MMTV LTR have failed to uncover any sequence-specific negative elements that could explain the strong transcriptional stimulation observed here (3–6, 24). We conclude that the observed negative control of the MMTV promoter most likely is caused by chromatin organization.

It is conceivable that some of the factors interacting with regulatory DNA segments in the MMTV LTR are required to relieve the promoter from the suggested chromatin repression under normal in vivo conditions. Additional mechanisms, such as protein-protein contacts between activating domains of regulatory DNA binding proteins and other factors of the transcription machinery, are evidently utilized in transcriptional regulation (2, 14, 15, 25). Such mechanisms probably contribute to the glucocorticoid-induced level of transcription also in the MMTV promoter. We have attempted to determine whether GR has such effects by coinjecting GR expression vector (HGI) together with MMTV test plasmid and competitor to see whether this results in increased transcription compared to when only competitor DNA is injected. In such experiments, no drastic increase in MMTV transcription was seen in the presence of receptor and hormone (data not shown). This result does not exclude any such positive effects by GR; a careful quantitation of the hormone-induced transcription compared with the increased basal level caused by the competitor DNA has been difficult to make since the competitor DNA also has a general repressing effect on transcription (Fig. 5). Furthermore, in our experiments there was a considerable variability in the level of hormone induction of MMTV transcription in the oocytes (data not shown), making it difficult to demonstrate any small positive effect by GR and hormone on MMTV transcription in addition to the induction caused by injection of competitor DNA.

Our experiments demonstrate that the promoter is negatively regulated in vivo and provide direct support for the hypothesis that nucleosomes play an active role in this regulation. Since the MMTV promoter does not require replication for induction to occur (33), transcriptional activation, according to this hypothesis, would require a nucleosome displacement activity. An interesting possibility is that such a function resides in GR.

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