Nucleosome-Mediated Disruption of Transcription Factor-Chromatin Initiation Complexes at the Mouse Mammary Tumor Virus Long Terminal Repeat In Vivo

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Glucocorticoid induction of mouse mammary tumor virus (MMTV) is short lived, returning to base levels within 24 h despite the continued presence of hormone. MMTV DNA sequences assembled as chromatin require hormone for binding by nuclear factor 1 (NF1) and octamer proteins (OCT). However, in the same cells, NF1 and OCT factors are bound to transiently introduced DNA in the absence of hormone. In contrast, recruitment of the TATA-binding protein and a novel DNA-binding protein, which we have designated F_{DT}, for factor downstream of the TATA-binding protein, is hormone dependent for both stable and transient templates. Furthermore, transient DNA templates, but not nucleosomal templates, retain these transcription factors over the course of 24 h. This finding suggests that MMTV chromatin structure contributes to activation and cessation of transcription in vivo.

The regulation of transcription requires the complex interaction of sequence-specific transactivating proteins as well as the extensive array of basal transcription factors without sequence-specific binding capacity (24, 58). These interactions are knit together by an increasingly large number of coactivator proteins which appear to facilitate contact between the enhancer or repressing proteins on this basal machinery (16, 30, 40, 59). In the nucleus of eukaryotes, this process is further complicated by the fact that the target sites of *trans*-acting factors are often assembled as chromatin (18, 51). Not surprisingly, this compaction of DNA sequences produced by the association of histones has been shown to be relevant to the regulation of transcription for several eukaryotic genes in vivo (17, 18, 51). A particularly well studied group are the inducible promoters as exemplified by the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (6, 57), the *PHO5* gene in *Saccharomyces cerevisiae* (1), and the tyrosine aminotransferase gene in liver (12, 42). While recent experiments analyzing the *Drosophila* *HSP26* and the chicken vitellogenin genes have suggested a positive potentiation for chromatin (29, 44), in most cases its effect has been to inhibit transcription (1, 6, 50).

The significance of a repressive function of chromatin structure has been amply demonstrated with genetic and biochemical experiments (15, 19, 37). Genetic studies demonstrated that production of yeast strains deleted of specific histones and/or with specific mutations to prevent posttranslational modifications results in profound effects on regulated gene expression. In one case, Simpson and colleagues demonstrated that the ability of certain transactivating factors such as the $\alpha 2$ repressor to influence nucleosomal positions

is dependent upon interactions with the tails of histone H4 (43).

Biochemical studies using in vitro assembly of a variety of promoter sequences into chromatin have demonstrated a general inhibitory role for nucleosomes in transcription (15, 23, 27, 56). However, the prebinding of transcription factors, such as the transcription factor IID (TFIID) complex, is sufficient to overcome this effect (55). A variety of elegant in vitro studies have demonstrated that the prior assembly of these sequences into nucleosomes is sufficient in many cases to preclude the binding of some but not all transcription factors (7, 54).

As a model system with which to examine the contributions of chromatin structure to transcription, we have used the MMTV LTR (4). When stably introduced into rodent cells, the MMTV LTR acquires a phased array of nucleosomes that encompass the promoter (34, 41). The second nucleosome of this phased array (nucleosome B) contains within its borders binding sites for the glucocorticoid receptor as well as nuclear factor 1 (NF1) and is adjacent to binding sites for the octamer factor (OCT) and the TFIID complex that includes the TATA-binding protein (TBP) (10, 22). Upon hormonal stimulation, the preinitiation complex, which consists minimally of the transcription factor NF1 and the TFIID complex, is rapidly observed in vivo on the promoter (8, 14). This loading of transcription factors is coincident with the initiation of transcription and hypersensitivity to a variety of agents such as restriction enzymes, DNase I, and MPE [methidiumpropyl-EDTA-Fe(II)] (7, 41, 57), and it has been correlated to the loss of and/or remodelling of nucleosome B (21).

Analyses of these phenomena have been greatly enhanced by our ability to reconstitute fragments of the MMTV promoter in vitro and to assess the ability of transcription factors to interact with and stimulate transcription. Guided by in vivo observations, a series of in vitro experiments have demonstrated that the prior assembly of portions of the MMTV LTR, either as mononucleosomes or as dinucleo-

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somes, restricts the binding of transcription factors such as NF1 but permits binding of glucocorticoid receptor (7, 36, 39). These studies, in agreement with ones carried out on the *Xenopus* 5S gene, synthetic *GAL4* promoters, and the adenovirus major late promoter, demonstrate a primary and repressive role for chromatin structure (23, 53).

We have recently demonstrated that when transiently introduced into rodent cells and examined in a time frame that does not allow the formation of a specific chromatin structure, the MMTV LTR is bound by NF1 in the absence of glucocorticoid. Subsequent addition of glucocorticoids did not induce any further increase in binding by NF1 (8). These experiments provide a direct link between the previous *in vivo* and *in vitro* experiments describing NF1 and MMTV interactions. Further, the well-documented hormone-induced hypersensitivity to restriction enzymes was absent in these transiently introduced templates. However, despite the continued presence of NF1, the transiently introduced template remained hormone inducible. The ability of these transient templates to respond to hormones was attributed to the putative hormone-dependent loading of TBP on these templates (8).

Previous studies have focused almost entirely on the initiating events whereby the chromatin structure is altered to allow the binding of basal transcription factors (7, 54). In the studies presented here, we examined the end point of transcription to determine the role a specific chromatin structure may play in the cessation of transcriptional activity from a promoter *in vivo*. Our experiments suggest that the association of the preinitiation complex with the promoter is transient and maximal at 1 h and is lost after 24 h of continuous hormone treatment. In contrast, transiently introduced templates are not assembled into a specific chromatin structure and remain transcriptionally competent within the same time period. These experiments argue that the ability of a promoter to cease active transcription may, in certain cases, be regulated by the re-formation of chromatin.

MATERIALS AND METHODS

Cell lines and plasmids. Cells lines 1471.1, 433.33, and 2305 are bovine papillomavirus (BPV) transformants of mouse C127 cells with the MMTV LTR driving the chloramphenicol acetyltransferase (CAT) gene (1471.1 and 2305) or the *ras* oncogene (433.33). Cell lines were maintained in the presence of 10% fetal calf serum in Dulbecco modified Eagle medium as described previously (35). Activation of transcription was initiated with dexamethasone at a concentration of 10^{-7} M for the times indicated for the individual figures. Plasmid pM50 contains a fragment of the MMTV LTR from positions -223 to +107 cloned into plasmid pSP65 (7). Plasmid pLTRluc contains a full-length MMTV LTR driving the luciferase gene (28). Plasmid pHHCAT contains sequence identical to that of pM50 adjacent to the bacterial CAT gene (33).

In vivo chromatin analysis and transcription factor loading. Isolation of nuclei, digestion with restriction enzymes, and exonuclease III (Exo III) footprinting were carried out precisely as described previously (7, 48), with one exception. The removal of single-strand DNA following Exo III treatment was accomplished with mung bean nuclease under conditions specified by the manufacturer (Bethesda Research Laboratories). Analysis of *in vivo*-cleaved DNA and footprinting experiments were carried out by linear PCR as described previously (8). The purified products were separated on 7% acrylamide gels, subjected to autoradiography

at -70°C , and then visualized with Kodak XAR film. Analysis of transiently transfected DNA templates was accomplished as indicated above except that cells were transfected by using a standard calcium phosphate procedure and 5 μg of DNA per plate (8, 49).

RNA isolation, labelling of oligonucleotides, and primer extension. Total cytoplasmic RNA was prepared by lysing cells in a buffer containing 200 mM Tris-HCl, 140 mM NaCl, 2 mM MgCl_2 , and 0.5% Nonidet P-40. The cytoplasmic extract was adjusted to 0.1% sodium dodecyl sulfate and 5 mM EDTA and then subjected to multiple phenol and chloroform extractions followed by ethanol precipitation. Nucleotide sequences of oligonucleotide primers for MMTV and actin were 5'-TCTGGAAAGTGAAGGATAAGTGACGA-3' and 5'-ACCAGCGCAGCGATAATCGCCATCCAT-3', respectively. Oligonucleotides were prepared on an Applied Biosystems (Foster City, Calif.) 380A DNA synthesizer. Oligonucleotides were labelled with [^{32}P]ATP (6,000 Ci/mmol; New England Nuclear), using T4 polynucleotide kinase, and primer extension was carried out as described previously (9). Primer extension analysis of total RNA was performed with single-stranded ^{32}P -labelled oligonucleotides to yield extension products of 85 bp (MMTV; Fig. 1B, lanes 4 to 6) and 106 bp (actin; Fig. 1B, lanes 1 to 3). The results presented were obtained with RNA isolated from 433.33 and 1471.1 cells subjected to hormone treatment for 1 to 24 h, as indicated.

CAT assays. Cells, treated with hormone as described in the figure legends, were transiently transfected with 10 μg of reporter plasmid pHHCAT (31) as described previously (8). After 24 h, cells were harvested and a cytoplasmic extract was prepared. Activation of the transiently transfected CAT plasmid was assayed by standard procedures in a kinetic assay (32).

RESULTS

Kinetics of transcriptional induction of the MMTV promoter. Previous experiments (47, 57) have demonstrated that glucocorticoids induce an extremely rapid transcriptional response from the MMTV promoter *in vivo*. In the past, we have taken advantage of these observations to examine early time points (0.5 to 1 h) of hormone stimulation with respect to the formation of the preinitiation complex *in vivo* (8, 14). During the course of experiments designed to examine the stability of the transcription initiation complex on this promoter, we observed that when mouse 433.33 cells, containing the MMTV LTR driving the *ras* oncogene, are treated with glucocorticoid, there is a rapid induction of RNA transcription that peaks at 1 h and falls to near basal levels by 24 h (5). In this case, the levels of MMTV-initiated mRNA, as monitored by primer extension, peak at approximately 4 h of hormone treatment and decline to near basal levels within 24 h of continued hormone treatment (Fig. 1A, lanes 1 to 3). To eliminate contributions that may result from the instability of the *ras* mRNA assayed or contributions of the Ras protein (25), we carried out a similar experiment with a cell line producing the CAT mRNA from the MMTV LTR. The results (Fig. 1B, lanes 4 to 6) are identical to those seen with the MMTV-*ras* transcript (Fig. 1A, lanes 1 to 3). This increase is specific for the MMTV promoter, as no changes are observed with actin mRNA levels in these cells (Fig. 1B, lanes 1 to 3).

These results suggested that transcription from the promoter ceased or was severely diminished upon 24 h of hormone treatment. Control experiments demonstrated that

FIG. 1. Kinetics of MMTV induction monitored by mRNA accumulation. (A) Mouse 433.33 cells, with MMTV driving the *ras* oncogene, were treated with (+) or without (-) dexamethasone (10^{-7} M) for the times indicated, and then RNA was isolated. Levels of MMTV transcripts were analyzed with sequence-specific primers as described previously (9). Lane M, *Hae*III-digested ϕ X174 DNA as a marker. (B) Mouse 1471.1 cells, with MMTV driving the bacterial CAT gene, were treated with (+) or without (-) dexamethasone (10^{-7} M) for the times indicated, and then RNA was isolated. Levels of actin and MMTV transcripts were analyzed with sequence-specific primers as for panel A. Sizes are indicated in nucleotides.

dexamethasone was not metabolized during this time period, eliminating the loss of active hormone as an explanation for this result (5). Further, the presence of active hormone in the media after 24 h is demonstrated in the data presented in Fig. 5A and 6 (see below).

A signature response, to hormone, of the MMTV promoter is the appearance of a hypersensitivity to restriction enzymes coupled with the formation of the active transcription initiation complex (14, 57). We examined changes in chromatin structure that might occur within the same time frame as these changes in mRNA accumulation and initiation by use of a restriction enzyme accessibility assay *in vivo*. Coincident with this mRNA accumulation profile (Fig. 1), changes in chromatin structure in response to hormone examined by *Mbo*I and *Dde*I cleavage *in vivo* revealed a similar pattern of elevated cutting at 1 h of hormone treatment (Fig. 2A; compare lanes 4 to 6 and 7 to 9). Control experiments examining *Hae*III cleavage, which we have previously shown to be unaffected by hormone, demonstrated no difference over the time of any of the experiments (Fig. 2A, lanes 1 to 3). These experiments suggested that 24 h of glucocorticoid treatment resulted in the opening and closing of the promoter (Fig. 2A; compare lanes 3, 6, and 9).

In the next series of experiments, we directly examined the impact of this closing of chromatin structure at 24 h by assessing the stability of the preinitiation complex that forms in response to hormone treatment (Fig. 2B). An examination of the preinitiation complex *in vivo*, as monitored by NF1

loading, revealed that hormone stimulation led to a rapid appearance of NF1 at 1 h but not at 24 h. This result is consistent with the aforementioned kinetics of mRNA accumulation and restriction enzyme hypersensitivity (Fig. 1 and 2A). Thus, the MMTV promoter *in vivo* is responsive to glucocorticoids and undergoes a transient transactivation but then proceeds to become refractory to further stimulation despite the continued presence of hormone. The failure of the promoter to respond at 24 h of hormone treatment is correlated with the loss of hypersensitivity at this time point as well as with a subsequent failure to detect the preinitiation complex at 24 h of hormone treatment. (We use the term preinitiation complex to include NF1, OCT, TBP, and other members of the basal transcription machinery.)

Transcription preinitiation complex stability on nucleosomal and nonnucleosomal templates *in vivo*. We performed a series of experiments aimed at elucidating the mechanisms responsible for this transient activation. Two models were considered: (i) that the transient nature of the response was a result of alterations in either levels or activities of the component transcription factors (i.e., NF1, TFIID, and glucocorticoid receptor) and (ii) that the transient nature of the preinitiation complex was due to alterations in chromatin structure (i.e., re-formation of the nucleosome, thereby eliminating the preinitiation complex).

Our experimental scheme uses transient transfection of a reporter plasmid driven by the MMTV promoter of the same sequence as that contained within the cells on a BPV plasmid

FIG. 2. Kinetics of hormone-induced hypersensitivity and NF1 loading in an MMTV-BPV stable cell line. (A) In vivo hypersensitivity. Mouse mammary cells (433.33 cells) containing the MMTV promoter, mobilized on a BPV-based multicopy plasmid as a stable replicating unit, were treated with dexamethasone (Dex; 10^{-7} M) for 0, 1, and 24 h. Nuclei were isolated and subjected to *Hae*III, *Mbo*I, or *Dde*I digestion as described previously (7). The DNA was purified, and the *Mbo*I- and *Dde*I-digested samples were subjected to a second *Hae*III digest in vitro. The samples were then analyzed by primer extension with *Taq* polymerase (8). (B) Transcription factor loading in vivo. 433.33 cells were treated with dexamethasone (10^{-7} M) for 0, 1, and 24 h. The nuclei were isolated and digested with *Hae*III and Exo III, and then the purified DNA was analyzed by primer extension with *Taq* polymerase as described previously (8). The extension products were purified and then analyzed on a 7% sequencing gel before autoradiography at -80°C . The arrow indicates the NF1 binding site. Lane G, dideoxy G sequencing reaction.

FIG. 3. Experimental design to assay DNA-protein interactions on stable chromosomal and transiently transfected DNA templates. Murine cell lines stably transformed with high-copy-number BPV-MMTV plasmids are transiently transfected with plasmids containing the MMTV LTR driving an alternative reporter gene and/or with unique restriction enzyme sites. This allows the transient DNA to be distinguished from the endogenous chromosomal template by selection of primers or restriction enzyme sites. This is feasible since control experiments with Exo III digestion in the absence of restriction enzyme digestion to provide an entry site for Exo III fail to produce specific Exo III stops. Alternatively, the use of primers specific for the luciferase (Luc) and CAT genes, respectively, allows the independent analysis of transcription factors bound to the chromatin and transiently transfected templates, using DNA from the same nuclei.

but driving a different reporter gene (Fig. 3). The use of different reporter genes allow the two templates to be examined by a PCR-Exo III in vivo footprinting assay that distinguishes transient from stable templates. Plasmids are introduced by transient transfection at various times following hormone treatment to assess differences with respect to transcription factor levels and/or modifications which occur in response to hormone. As an example, cells containing endogenous copies of the LTR-CAT construct were treated for 24 h with hormone, at which time the endogenous promoter is refractory to stimulation, and then transfected with the transient LTR-luciferase template. The ability of the preinitiation complex to form and activate transcription from this promoter was then assessed.

As a prerequisite for these experiments, we determined whether the activation process was influenced by the calcium phosphate transfection protocol. To do this, we transiently transfected cells for periods as short as 1 h in the presence of dexamethasone and examined (i) whether the transfection process influenced the hormone inducibility of the resident stable template and (ii) whether transcription factors were loaded onto a newly introduced template. The results from one such experiment examining hormone-induced hypersensitivity are shown in Fig. 4A. At 1 and 4 h after transfection, the stable template, in the presence of calcium phosphate precipitate, demonstrated a hormone response, as visualized by the increased accessibility to *Mbo*I (Fig. 4A; compare lanes 1 and 2 and lanes 3 and 4).

Next, the ability of the hormone to promote loading of

FIG. 4. Hormone induction of hypersensitivity and transcription factor binding following short-term transfection. (A) In vivo restriction digestion of chromatin following transient transfection. 1471.1 cells were transfected with plasmid pLTRLuc (5 μ g) for either 1 or 4 h and then treated with or without dexamethasone (Dex; 10^{-7} M) for 1 h prior to harvesting of cells. The nuclei were isolated and digested with *Mbo*I, and the purified DNA was analyzed by primer extension with *Taq* polymerase as described previously (8). The extension products were purified and then analyzed on a 7% sequencing gel before autoradiography at -80°C . Lane M, *Hae*III-digested ϕ X174 DNA as a marker. (B) NF1 loading on chromosomal DNA (Ch) and a transiently transfected DNA (TT-DNA). Cells were transfected as in panel A and treated with or without dexamethasone (Dex) for 1 h, and the nuclei were isolated and then subjected to an *Hae*III-Exo III footprinting assay. The results shown in lanes 5 to 8 demonstrate the hormone-independent loading of NF1 detected after 1 and 4 h of transfection time compared with the hormone-dependent loading on the chromatin template (lanes 1 and 2). Lanes 3 and 4, dideoxy G and C sequencing reactions, respectively; lane 9, *Hae*III-digested ϕ X174 DNA as a marker. The fast migration of NF1 in lane 5 results from excess salt; we also observed the fast migration of the parental band at the top of the gel.

transcription factors in the newly transfected cells was examined. In vivo footprinting experiments revealed that transient transfection of the mouse cells does not inhibit or influence the steroid-induced transcription factor loading on the chromatin template (Fig. 4B; compare lanes 1 and 2). Thus, within 1 h of transfection, cells are able to exhibit a full hormone response, as indicated by changes in chromatin structure and the assembly of a preinitiation complex on stable templates. Significantly, the transiently transfected template was fully occupied by NF1 at this time, indicating that binding of this transcription factor was rapid and stable with or without hormone (Fig. 4B; compare lanes 5 to 9).

In subsequent experiments, we examined whether transcription factors were modified and/or lost after 24 h of hormone treatment. This was accomplished by treating cells for 0 or 23 h with dexamethasone, subsequently transfecting them with an MMTV LTR plasmid for 1 h, and then isolating the nuclei and examining the transcription factor complex

FIG. 5. Hormone-independent loading of NF1 on transiently transfected DNA after 1 and 24 h of transfection with and without hormone treatment. (A) 2305 cells were treated with dexamethasone (Dex; 10^{-7} M) for 0, 1, and 24 h and transfected with plasmid pM50 (5 μ g) for 1 h prior to harvest. The nuclei were isolated and digested with *Eco*RI and Exo III. The DNAs were purified and subjected to a second digestion with *Eco*RI. Exo III digestion products are specific for the transfected copy of the LTR because the chromatin template was not cleaved by *Eco*RI and therefore was unable to provide a specific entry site for Exo III. The purified DNA was analyzed by primer extension with *Taq* polymerase. The extension products were purified and analyzed on a 7% sequencing gel before autoradiography at -80°C . The arrow indicates the NF1-specific Exo III stop. Lane G_{seq}, dideoxy G sequencing reaction. (B) 2305 cells were treated with dexamethasone (Dex; 10^{-7} M) for 0.5 h and 24 h, as indicated. The cells were transfected with plasmid pM50 for 24 h prior to harvest. The nuclei were isolated and digested with *Eco*RI and Exo III. The purified DNA was analyzed by primer extension with *Taq* polymerase. The extension products were purified and analyzed on a 7% sequencing gel before autoradiography at -80°C . The arrow indicates the NF1-specific Exo III stop.

formed in vivo on the transient templates. The results (Fig. 5A) demonstrate that NF1 was present on the template under all conditions examined. Thus, NF1 was available and able to bind despite 24 h of continuous hormone treatment. This result demonstrates that for the duration of hormone treatment, NF1 is present and available for binding to the LTR but restricted in this association because of the formation of a specific chromatin structure. Further, these data can be interpreted as providing support for the second of the two alternative models proposed earlier; i.e., the transient nature of the preinitiation complex was due to alterations in chromatin structure (i.e., re-formation of the nucleosome, thereby eliminating the preinitiation complex).

As an alternative, cells were transfected for 24 h, and then hormone was added at the same time or 23.5 h later, to provide samples that had been subjected to 24 or 0.5 h of hormone treatment and 24 h of transfection. The results obtained are identical to those obtained in the previous transfection protocol, namely, that NF1 was able to bind in the absence of hormone and remained bound after subsequent treatment (Fig. 5B). The results in Fig. 4 both confirm and extend our previous observations on transcription factor loading on transiently transfected templates *in vivo* (8). These new data reveal that NF1 is present and able to bind DNA after 24 h of hormone treatment, and they suggest that it is prevented from binding to its cognate site by the reestablished nucleosome B.

Hormone-independent and -dependent loading of OCT and TBP *in vivo*. In addition to the hormone-dependent binding of NF1 which corresponds to the 5' Exo III boundary, the 3' Exo III boundary of the preinitiation complex extends to -6 on the MMTV promoter (14). *In vitro* experiments suggested that the 3' stop results from components of a partially purified TFIID fraction (13). The experiments presented here take advantage of a cell line that contains an MMTV-BPV chimera with a unique *Bam*HI restriction site downstream of the transcription initiation site at position +123 relative to the start site of transcription. The advantage of this approach is that it allows one to monitor the appearance of the transcription factors, including TBP and OCT, that bind 3' to NF1. The results shown in Fig. 6A demonstrate the formation of just such a preinitiation complex. In this case, we observed hormone-dependent stops that mapped to the binding sites for NF1, both the proximal and distal OCT binding sites as well as the TBP binding site (Fig. 6A, lane 2). These experiments are the initial observation of these components (OCT and TBP) of the transcription initiation complex on the stable chromatin template. They also demonstrate that like NF1, the other members of the complex are present at 1 h of hormone treatment and then absent at 24 h.

We compared this pattern of hormone responsiveness on MMTV chromatin (Fig. 6A) with that observed on the transient template (Fig. 6B). The results show that NF1 is bound constitutively in the absence of hormone and is unaltered at 1 and 24 h of hormone treatment. In addition, we are now able to observe the binding of the OCT proteins, both distal and proximal, in the presence or absence of hormone. This demonstrates that they, like NF1, are able to bind to DNA if their sites are not assembled as chromatin. This is not the case for TBP binding, which is strictly hormone dependent on the transiently transfected molecules, being observed only after hormone treatment. Finally, we observed the hormone-dependent binding of a novel transcription factor, designated F_{DT} for downstream of TBP, to the transient template (Fig. 6B, lanes 2 and 3). This factor is similar to TBP in that its binding is also hormone dependent on the transient template. We are currently in the process of cloning this factor to ascertain how it may function in this regulatory cascade.

Our ability to detect multiple factors in these Exo III footprinting experiments was initially surprising and may result from one of two possibilities. In the first case, it may result from heterogeneity of the protein-DNA complexes on the multiple templates present in these cells. Alternatively, it may stem from the ability of Exo III nuclease to displace a fraction of the bound F_{DT} , TBP, and OCT during the digestion. We favor the second possibility for two reasons. First, in experiments in which the Exo III entry site is 5' to NF1 (Fig. 2B, 4B, and 5), we detect only NF1. If the

FIG. 6. Detection of a preinitiation complex on stable and transiently transfected MMTV DNA *in vivo*. (A) Chromatin. 433.33 cells were treated with dexamethasone (Dex) for either 0, 1, or 24 h. The nuclei were isolated and digested with *Bam*HI and Exo III. The DNA was purified and analyzed by PCR. The primer used, 5'-TTAAGTAAGTTTTGGTTACAACT-3', corresponds to positions -200 to -176 of the MMTV LTR. The extension products were purified and analyzed on a 7% sequencing gel before autoradiography at -80°C. Lane G, dideoxy G sequencing reaction. Positions of Exo III-dependent stops and of the relevant transcription factors, NF1, OCT (distal [OCT_D] and proximal [OCT_P]), and TBP. (B) Transiently transfected DNA. 2305 cells were treated with dexamethasone (10^{-7} M) for 0, 1, and 24 h. The cells were transfected with plasmid pM50 for 1 h prior to harvest. The nuclei were isolated and digested with *Hind*III and Exo III. Exo III digestion products are specific for the transfected copy of the LTR because the chromatin template was not cleaved by *Hind*III and therefore was unable to provide a specific entry site for Exo III. The purified DNA was analyzed by primer extension with *Taq* polymerase. Exo III-dependent stops are indicated for the relevant transcription factors, NF1, OCT, TBP, and F_{DT} . Lane 1, dideoxy G sequencing reaction.

templates represented a heterogeneous population, we would anticipate that we would also observe OCT and TBP in these experiments. The second reason results from the high-affinity binding of NF1 to its site. In all of the experiments reported here and described previously, we have never observed Exo III digestion through the NF1 site, suggesting that it functions as a much more efficient block to Exo III than other transcription factors bound at the MMTV promoter, such as OCT and TBP. This inference is further supported by the data in Fig. 6, which demonstrate that the last factor detected on the promoter is a 3' boundary for NF1.

FIG. 7. Hormonal induction of transiently transfected templates. 433.33 cells, which contain an MMTV-*ras* reporter, were transiently transfected (TT) with plasmid pHHCAT (10 μ g) and treated with dexamethasone (Dex) for an additional 24 h. The cells were harvested, and a cytoplasmic extract was made to determine CAT activity for the various conditions. A representative experiment is shown; each datum point represents the average of duplicates. Similar results were obtained in three independent experiments. Three conditions were examined: naive cells, no hormone treatment and transfection for 24 h (■); naive cells, dexamethasone treatment for 24 h and transfection for 24 h (▲); and cells pretreated with dexamethasone for 24 h prior to transfection and then maintained in dexamethasone for 24 h with transfection for 24 h (●).

These results allow us to separate the *trans*-acting factors which bind at the LTR into two groups: (i) those whose binding is hormone dependent, i.e., TBP and F_{DT} , and (ii) hormone-independent NF1 and OCT. However, in contrast to what is observed on the chromatin template, TBP and F_{DT} remain bound at 24 h of hormone treatment. Thus, the establishment of a specific chromatin structure both prevents the promiscuous binding of transcription factors and removes them to establish the basal state.

Our Exo III footprinting assay does not reproducibly detect the glucocorticoid receptor bound to DNA, suggesting that the binding of the receptor to its target either is transitory (i.e., a hit-and-run mechanism) or is labile to this nuclease (8, 14). However, others (38) have suggested that the steroid receptor, once bound to hormone, remains in the nucleus under a similar time frame. While we do not address the issue directly, our data on the hormone-dependent loading of TBP and F_{DT} (Fig. 6) and the results presented below indicate that this hormone-dependent response is active in cells that were treated continuously for 24 h.

Activation of transient templates in refractory cells. To assess the functional significance of the transcription factor loading that we observed following 24 h of hormone treatment, we carried out a series of CAT assays. For these experiments, cells that had been treated with dexamethasone for 0 or 24 h were transfected with the expression vector pHHCAT for a period of 24 h (31). The cells were then treated with or without hormone for an additional 24 h to yield populations of cells that had never received hormone for 24 h in conjunction with transfection or received hormone for a total of 48 h, 24 h prior to transfection and for the duration of the transfection. Results of these experiments (Fig. 7) indicate that a newly introduced reporter plasmid is transcriptionally competent in a background of cells wherein the endogenous MMTV promoter is refractory to stimulation by glucocorticoids. These data establish that the loading of transcription factors observed on the transiently introduced

template foreshadows an active preinitiation complex capable of initiating transcription from these templates.

DISCUSSION

Our results demonstrate that the ability to disrupt the transcription initiation complex *in vivo* is observed only on stable chromatin templates and not on transiently transfected DNA. The experiments outlined here reveal a direct correlation between kinetics of chromatin hypersensitivity and the loading of transcription factors in the MMTV system. Furthermore, these studies divide the factors that interact with the promoter into two classes based on the requirement for glucocorticoid receptor action. Finally, they argue that certain features of both the activation as well as the termination of active transcription cannot be modelled on templates which do not form stable nucleosomal structures.

Chromatin versus nonchromatin templates *in vivo*. The importance of chromatin structure in limiting the access of transcription factors has been explored in experiments using a variety of yeast promoters (20). In the case of the *PHO5* locus, a tight correlation between the activation of transcription and displacement of nucleosomes has been reported (2). In higher eukaryotes, the MMTV LTR has provided an outstanding model with which to analyze this process (21). The administration of glucocorticoids to mouse cells containing the MMTV LTR results in a rapid induction of transcription that can be tightly correlated with hypersensitivity to endonucleases and restriction enzymes (4). Concomitant with this hypersensitivity is a hormone-dependent loading of transcription factors to form a preinitiation complex that consists of, but not exclusively, NF1 and TBP (14). The exclusion of transcription factors observed *in vivo* prior to the administration of hormone can be mimicked *in vitro* by reconstitution of this DNA as chromatin and the subsequent addition of purified transcription factors (7).

The contribution chromatin structure makes to MMTV regulation was analyzed *in vivo* by transiently introducing MMTV plasmids into mouse cells that contain an endogenous copy of the LTR. By the use of sequence-specific primers, we were able to assess the structure of the newly introduced molecule and to characterize its interactions with transcription factors. These studies demonstrated that the transiently transfected templates had features significantly different from those of the endogenous copy. Under the conditions examined, the transiently transfected DNA was not assembled as specifically phased nucleosomes and showed constitutive sensitivity to a variety of restriction enzymes. In these kinetic studies, NF1 was constitutively loaded on the transiently transfected DNA in the absence or presence of hormone, while the chromatin template exhibited the hormone-dependent loading and subsequent unloading of NF1 in the same cells. Our results indicate that binding of the OCT protein is similar to that of NF1 in that the protein is able to bind independently of hormone treatment if its site is not assembled into chromatin.

The requirement for hormone in the presence of constitutively loaded NF1 and OCT to stimulate transcription is indicated by the fact that TBP binding was strictly hormone dependent for both the stable template and the transiently introduced template. Further, we have detected the hormone-dependent binding of an additional transcription factor, which we have termed F_{DT} , downstream of TBP (3' Exo III boundary at -24) with a 3' Exo III boundary at -10. The sequence from the 3' boundary of F_{DT} to the TBP binding

site does not correspond to any known regulatory element. A preliminary search of the GenBank data base reveals a 100% match of 11 bases with human calbindin D_{9k} gene; the significance of this match is unknown at present (26). However, it is intriguing that calbindin D_{9k} is also a steroid hormone (estrogen)-responsive gene, which may suggest that F_{DT} is involved in steroid hormone response pathways. It is worth recalling that the boundaries of nucleosome A lie outside the region of hypersensitivity to endonucleolytic agents with a 5' boundary at -25 (7, 11). This suggests that in chromatin, the binding of TBP must occur immediately adjacent to and the binding of F_{DT} must occur directly on this nucleosome upon hormone stimulation.

In a related series of experiments, we have attempted to determine whether we could overcome this refractory phase by use of an alternative hormone ligand (5, 46). In these experiments, transfection of a chicken progesterone receptor into mouse cells failed to alter the kinetics of the response. Subsequent experiments demonstrated that this failure is due to the inability of the newly introduced progesterone receptor to modulate the MMTV promoter in a chromatin context. Control experiments using transient transfection demonstrated that the receptor introduced in the cells was capable of activating the transient template while being unable to activate the stable template. These experiments are consistent with the results reported here in suggesting that certain attributes of the MMTV promoter in chromatin are not reproducibly observed when these sequences are transiently transfected into mouse cells.

These studies link the closing or loss of hypersensitivity and the loss of transcriptional activity to the assembly of the promoter as chromatin in a manner that has been found previously for the initiation of transcription. In this respect, these results are reminiscent of observations for the *Xenopus* 5S gene in vitro (3). In their experiments, Almouzni et al. (3) demonstrate that preexisting transcription complexes of 5S DNA are disrupted by the assembly of physiologically spaced nucleosomes on the promoter. The experiments of Jaggi et al. (25) have demonstrated a role for oncogenes such as *ras* and *mos* in repressing transcription from glucocorticoid-responsive promoters such as the MMTV LTR. The proposed mechanism involves the down-regulation of the activated receptor, resulting in a transient activation similar to the one reported here. The fact that we observe the same kinetics in cell lines that do not express these oncogenes (Fig. 1B, 5, and 6B) suggests that this is an intrinsic feature of this promoter as chromatin.

Our experiments have allowed us to eliminate the down-regulation or loss of specific transcription factor necessary for stimulation of mRNA synthesis of this promoter as a mechanism for the refractory period. They suggest that there may be a requirement for some activity or associated factor needed for the glucocorticoid receptor-mediated chromatin disruption or nucleosome displacement. This putative activity or factor would not be required for transcription from the transient template. This agent, which is required for the chromatin modification that is necessary for induction, would be what is rate limiting and lost or down-regulated after the initial chromatin disruption event. This would then prevent the continued activation or the reactivation of the promoter as seen at 24 h of hormone treatment.

One possibility may be some type of replication-mediated phenomenon as has been suggested for the *Xenopus* 5S gene (52). In this case, a competitive process occurs between the binding of transcription factor IIIA and histones to establish an inactive or active gene. Our transient transfection exper-

iments suggest that transcription factors NF1 and OCT bind first and prevent the assembly of the specific chromatin structure that is observed on stably introduced MMTV LTR sequences. This result is complementary to our previous experiments in which the binding of NF1 and the presence of nucleosome B are never observed on the same template; i.e., they are mutually exclusive. Thus, one possibility may be that as our transfected plasmids do not replicate, they are never able to displace NF1 and OCT to allow the histone octamer access to the transient templates. With respect to replication, our previous experiments suggest that replication per se is not necessary for this activation process; indeed, activation occurs quite efficiently if replication is blocked by the drug aphidicolin (6). We are currently addressing the question of whether inhibition of replication will have an effect on the kinetics of the response. A similar replication-independent activation of transcription has also been recently described for the *PHO5* gene (45).

We have demonstrated that concomitant with this closing of the promoter is the loss of the preinitiation complex. The loss of hypersensitivity that occurs upon prolonged hormone treatment is also coincident with the loss of activity from this promoter. Using transient transfection experiments, we have demonstrated that this loss of activity or refractory period is most likely related to the assembly of the promoter as chromatin. Transient transfection of MMTV reporter plasmids into cells previously treated with hormone demonstrates that the *trans*-acting factors necessary for efficient induction of transcription can be observed either functionally or by footprinting assays.

The binding of transcription factors NF1 and OCT to chromatin templates in vivo is hormone dependent in that it requires the glucocorticoid receptor-mediated disruption or displacement of the B (second) nucleosome, visualized as hypersensitivity to restriction enzyme, to bind. However, the fact that these proteins are bound to the transiently transfected DNA in the absence of hormone stimulation suggested that binding was not facilitated by the glucocorticoid receptor directly but rather by the availability of its site as a result of the disruption of chromatin structure. This contrasted with the TBP and F_{DT} binding, which occurred only in the presence of hormone on both templates, suggesting that it was a direct receptor-mediated event.

In summary, our experiments reveal that certain features of the activation as well as cessation of transcription from the MMTV LTR are strictly chromatin dependent and cannot be modelled on transiently transfected DNA. They also raise the possibility of the existence of a factor(s) that may contribute to the chromatin disruption which is not required for transcription from the promoter.

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