

# Nucleoprotein Structure Influences the Response of the Mouse Mammary Tumor Virus Promoter to Activation of the Cyclic AMP Signalling Pathway

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**Recent studies have provided evidence of crosstalk between steroid receptors and cyclic AMP (cAMP) signalling pathways in the regulation of gene expression. A synergism between intracellular phosphorylation inducers and either glucocorticoids or progestins has been shown to occur during activation of the mouse mammary tumor virus (MMTV) promoter. We have investigated the effect of 8-Br-cAMP and okadaic acid, modulators of cellular kinases and phosphatases, on the hormone-induced activation of the MMTV promoter in two forms: a transiently transfected template with a disorganized, accessible nucleoprotein structure and a stably replicating template with an ordered, inaccessible nucleoprotein structure. Both okadaic acid and 8-Br-cAMP synergize significantly with either glucocorticoids or progestins in activating the transiently transfected MMTV template. In contrast, 8-Br-cAMP, but not okadaic acid, is antagonistic to hormone-induced activation of the stably replicating MMTV template. Nuclear run-on experiments demonstrate that this inhibition is a transcriptional effect on both hormone-induced transcription and basal transcription. Surprisingly, 8-Br-cAMP does not inhibit glucocorticoid-induced changes in restriction enzyme access and nuclear factor 1 binding. However, association of a complex with the TATA box region is inhibited in the presence of 8-Br-cAMP. Thus, cAMP treatment interferes with the initiation process but does not inhibit interaction of the receptor with the template. Since the replicated, ordered MMTV templates and the transfected, disorganized templates show opposite responses to 8-Br-cAMP treatment, we conclude that chromatin structure can influence the response of a promoter to activation of the cAMP signalling pathway.**

The mouse mammary tumor virus long terminal repeat (MMTV LTR) has been used extensively as a model for both steroid-induced transcription and the effects of chromatin structure on transcription. The MMTV promoter can be activated by glucocorticoids, progestins, androgens, and mineralocorticoids through their individual receptors (8, 18, 21, 55). In chromatin, the MMTV LTR exists as an ordered array of six nucleosomes, one of which (Nuc-B) is associated with the promoter region containing binding sites for glucocorticoid receptor (GR) and nuclear factor 1 (NF1) (54). In the absence of activated GR, the MMTV chromatin template is found to be in a "closed" architecture, with the transcription factors NF1 and OTF1 largely occluded from their high-affinity binding sites (20, 35). Upon treatment with glucocorticoids, the Nuc-B region undergoes a structural transition which results in an opening of chromatin structure, characterized by increased nuclease accessibility and the binding of NF1, OTF1, and the basal transcriptional machinery (4, 20, 35, 54). These observations suggested a bimodal model of MMTV transcriptional activation (6), in which nucleoprotein structure limits the access of transcription factors to their sites on MMTV chromatin in the absence of hormone. This repression is overcome through a chromatin structural transition mediated by activated GR, which then actively participates in the stable formation of the initiation complex at the TATA box (6). Thus, the receptor acts bimodally to relieve repression by chromatin structure and to facilitate the formation of a stable and activated initiation complex. The derepression step of this pathway is not observed on transiently transfected MMTV templates for which restriction enzyme accessibility is unchanged by hor-

mone treatment, and NF1 and OTF1 are constitutively loaded in the absence of hormone (6, 35). Thus, in stably replicating MMTV templates, an ordered chromatin structure imposes an additional level of transcriptional regulation over that observed for transiently transfected templates, which have a relatively accessible nucleoprotein structure that may feature nucleosomes deposited in a disorganized fashion rather than the highly ordered array observed for the replicated templates (6).

There is now considerable evidence for crosstalk between various cellular signalling pathways and steroid receptors (reviewed in reference 42). Several laboratories have shown a functional antagonism between steroid receptors and the AP1 transcription factor, which is activated through a pathway involving protein kinase C (23, 32, 58, 64). This is thought to occur by at least two mechanisms, one involving protein-protein interactions independent of DNA (58, 64) and the other involving interactions at a given promoter (23, 32). There is also evidence of crosstalk between steroid receptor action and cyclic AMP (cAMP) signalling pathways. The glycoprotein hormone alpha-subunit gene provides an example of antagonism between activated CREB, a target of protein kinase A (PKA), and activated GR (2, 61). Although there are overlapping sites for both transcription factors on the promoter, GR binding to DNA is not necessary for inhibition of transcription in response to activation of CREB (61). This implies a mechanism which involves inhibitory protein-protein interactions rather than competition for DNA binding sites. In contrast, the phosphoenolpyruvate carboxykinase gene promoter is stimulated by both activated GR and CREB in an additive fashion (29). Binding sites for both factors are found in the promoter but at a distance from each other (30, 37); in fact, the presence of the CREB binding sequence has been reported to be necessary for the stimulatory response to glucocorticoids (29). It is

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evident from these studies that promoter context is important in determining whether the GR-CREB interaction is agonistic or antagonistic.

Activation of the cAMP signalling pathway results in a synergistic stimulation of the MMTV promoter in the presence of either activated GR or progesterone receptor (PR) (10, 22, 47, 53, 57). This has been shown in various cell types and is probably mediated through the proximal promoter (47). However, activation of the cAMP signalling pathway does not appear to result in changes in phosphorylation of the receptor itself (10, 47, 57), an observation which has led to the hypothesis that the ultimate target of PKA activation is a factor (or factors) which interacts with the receptor to induce transcription (9, 47, 49). In any case, these results imply that activation of the GR and the cAMP signalling pathway leads to more efficient interactions between the soluble transcription factors at the MMTV promoter.

We have studied the effects of 8-Br-cAMP (a PKA inducer) and okadaic acid (a phosphatase inhibitor) on hormone-induced activation of two types of MMTV templates, one transiently transfected and the other stably replicating. The former adopts a nucleoprotein structure that is highly accessible to nucleases and transcription factors, while the latter has the ordered, inaccessible nucleoprotein structure described above and requires activated GR or PR to acquire a more accessible state. We report here that 8-Br-cAMP treatment has opposite effects on the two types of template, synergizing with activated receptor on the transiently transfected template but antagonizing both basal transcription and receptor-induced transcription on the stably replicating template in the same cell line. These results strongly indicate that, in addition to the specific milieu of transcription factors at the MMTV promoter, the nucleoprotein structure of the promoter can influence its response to intracellular signals.

## MATERIALS AND METHODS

**Cell culture, transfections, and luciferase assay.** Cell lines 904.13 and 1470.2 were both derived from C127 mouse mammary adenocarcinoma cells and contain stably replicating copies of bovine papillomavirus (BPV)-MMTV LTR fusions. Cell line 904.13 contains a BPV-MMTV *ras* fusion and has been described previously (50). Cell line 1470.2 contains a BPV-MMTV chloramphenicol acetyltransferase (CAT) fusion derived from plasmid pM25 (3). Cell line 3017.1 was derived as a single-cell clone from 1470.2 cells after stable transfection with pRSVneo, a neomycin expression vector, and pcPRO, a chicken progesterone receptor expression vector. Cell line 1505 is derived from NIH 3T3 cells and has, in addition to several endogenous proviruses, a single integrated copy of an MMTV *ras* cassette downstream from the Harvey sarcoma virus enhancer (54a). All cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% charcoal-stripped serum. Transfections were carried out by the calcium phosphate precipitation method in six-well dishes using carrier DNA with 250 to 500 ng of pLTRluc (36). Hormone treatments were carried out for 24 h at a concentration of 100 nM. Okadaic acid (Calbiochem) and 8-Br-cAMP (Sigma) were used at concentrations of 50 nM and 1 mM, respectively. Cells were harvested and assayed for luciferase activity as previously described (36).

**RNA isolation and assay.** Cells were treated for 4 to 5 h with various effectors (at the concentrations indicated above unless otherwise indicated) and harvested by scraping. Total cellular RNA was isolated as described previously (7) and subjected to S1 nuclease assay. The probe used in these assays was generated by multiple rounds of *Taq* polymerase extension from antisense oligonucleotide primers (CAT sequence for 1470.2 and 3017.1 RNAs; MMTV sequence for 1505 RNA) using *Sst*I-digested pM25 as a template. After gel purification the probe ( $10^5$  cpm) was hybridized to RNA (10  $\mu$ g) in 40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.4)–200 mM NaCl–80% formamide–1 mM EDTA at 37°C. S1 nuclease (100 U per sample) digestions were carried out at room temperature for 1 h in 30 mM sodium acetate (pH 4.6)–50 mM NaCl–1 mM zinc acetate. Digestion products were electrophoresed on 8% denaturing urea-acrylamide gels (Sequal; National Diagnostics), which were dried and exposed to PhosphorImager screens. Visualization and quantitation of digestion products were carried out in a Molecular Dynamics PhosphorImager with Imagequant software.

**Nuclear run-on transcription assay.** Nuclei were isolated as described below and frozen in aliquots of  $3 \times 10^7$  to  $5 \times 10^7$  nuclei. Briefly, nuclei were thawed

on ice and washed once in 10 mM Tris (pH 8.0)–5 mM MgCl<sub>2</sub>–40% glycerol–2.5 mM dithiothreitol (DTT). Nuclear pellets were resuspended in 200  $\mu$ l of the wash buffer, to which an equal volume of reaction buffer (10 mM Tris [pH 8.0], 5 mM MgCl<sub>2</sub>, 200 mM KCl, 200 U of RNasin [Promega] per ml, 1 mM CTP, 1 mM GTP, 2 mM ATP, 2  $\mu$ M UTP, 5 mM DTT) was added. The reaction was started with the addition of 20  $\mu$ l [ $\alpha$ -<sup>32</sup>P]UTP (3,000 Ci/mmol; Amersham) and allowed to proceed at 26°C for 1 h. The reaction was terminated and the RNA was extracted by the addition of phenol-chloroform-isoamyl alcohol and RNazol (Teltest, Inc.), followed by precipitation by isopropanol. The RNA pellet was resuspended in 10 mM Tris (pH 8.0)–1 mM EDTA–0.1% sodium dodecyl sulfate (SDS) and reprecipitated with ethanol. The final RNA pellet was resuspended in TE (10 mM Tris [pH 8.0], 1 mM EDTA) and passed through a Sephadex G-25 spin column (Boehringer Mannheim). Labelled RNA was then diluted to  $3 \times 10^6$  cpm/ml in hybridization buffer (3 $\times$  SSC [1 $\times$  SSC is 0.15 NaCl plus 0.015 M sodium citrate], 20 mM sodium phosphate [pH 7.3], 0.02% polyvinyl pyrrolidone, 0.02% Ficoll, 0.1% SDS, 100  $\mu$ g of yeast tRNA [Life Technologies] per ml). Hybridizations were carried out at 60°C overnight.

Slot blots were prepared as follows. DNA fragments containing sequences from actin, MMTV *ras* (pM18), MMTV CAT (pM25), or pUC18 were isolated and denatured in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0)–1 mM EDTA–6% formaldehyde by boiling for 1 min followed by incubation at 60°C for 1 h. Fragments were then diluted with 20 $\times$  SSC and applied to a nylon membrane (Hybond; Amersham) in 1- $\mu$ g aliquots by using a slot blot vacuum apparatus (Hoefer). Membranes were exposed to UV light for 1 min in a Stratalink (Stratagene). Prehybridization was carried out overnight at 60°C in the same buffer as that for hybridization. After hybridization, membranes were washed for 30 min at room temperature in 2 $\times$  SSC–0.1% SDS, 30 min at 60°C in 2 $\times$  SSC–0.1% SDS, 60 min at 60°C in 0.5 $\times$  SSC–0.1% SDS, and 30 min at 60°C in 0.1 $\times$  SSC–0.1% SDS. Membranes were then dried and exposed to PhosphorImager screens.

**Isolation and digestion of nuclei.** Cells were washed and scraped into phosphate-buffered saline. After pelleting, cells were resuspended in HB (0.3 M sucrose, 2 mM magnesium acetate, 3 mM CaCl<sub>2</sub>, 10 mM HEPES [pH 7.8], 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1% Triton X-100) and Dounce homogenized (10 strokes, A pestle). The lysate was mixed with an equal volume of PB (25% glycerol, 5 mM magnesium acetate, 10 mM HEPES [pH 7.8], 5 mM DTT, 0.1 mM EDTA) and layered over a cushion of PB. Nuclei were pelleted through the cushion by centrifugation at 1,500 rpm for 15 min in a Sorvall RT6000D. The pellets were resuspended in NSB (25% glycerol, 5 mM magnesium acetate, 5 mM HEPES [pH 7.8], 0.1 mM EDTA, 0.5 mM DTT) at a ratio of 200  $\mu$ l of NSB per  $3 \times 10^7$  to  $5 \times 10^7$  nuclei and stored at –200°C.

For digestion of nuclei, nuclei were thawed on ice and washed once in NSB containing 2.5% glycerol. Nuclear pellets were resuspended in digestion buffer (2.5% glycerol, 1 mM MgCl<sub>2</sub>, 50 mM NaCl, 50 mM Tris [pH 8.0], 1 mM  $\beta$ -mercaptoethanol) at 0.75 to 1 mg of DNA per ml and divided into 100- $\mu$ l aliquots. Nuclei were then digested with various nucleases for 15 min at 37°C. The reactions were terminated with the addition of 5 volumes of stop buffer (10 mM Tris [pH 7.5], 10 mM EDTA, 0.5% SDS, 100  $\mu$ g of proteinase K per ml). Samples were then incubated overnight at 37°C. Nuclease concentrations (in units per milliliter) were as follows: *Sac*I, *Hae*III, *Bam*HI, and *Kpn*I, 1,000 each;  $\lambda$  exonuclease, 100 to 200.

**Analysis of DNA digested in vivo.** DNA from digested nuclei was diluted with an equal volume of TE, extracted once with phenol-chloroform-isoamyl alcohol and precipitated with ethanol. Pellets were air dried and resuspended in TE. Oligonucleotide primers were labelled with polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (NEN). Ten to twenty micrograms of DNA was mixed with labelled primer and subjected to linear amplification with *Taq* polymerase as previously described (6). Samples were then extracted once with phenol-chloroform-isoamyl alcohol and precipitated. Extension products were separated on 8% denaturing polyacrylamide gels, which were dried and exposed to PhosphorImager screens.

## RESULTS

**Experimental design.** We have compared the functional activities of two MMTV templates which adopt different nucleoprotein conformations in the cell. We have used multiple cell lines containing stably replicating copies of MMTV templates which are characterized by an ordered nucleosomal array (54) and a GR-induced structural transition, as measured by increased accessibility to nucleases (4, 6, 54, 65), loss of histone H1 (12), and increased binding of NF1, OTF1, and the initiation complex (20, 35). Into these cells we have transiently transfected another MMTV template, pLTRluc, which consists of a full-length MMTV LTR driving the luciferase gene (36). This template has a disordered nucleoprotein structure which is constitutively accessible to nucleases and to binding by NF1 and OTF1 (6, 35). Upon treatment with glucocorticoid there is

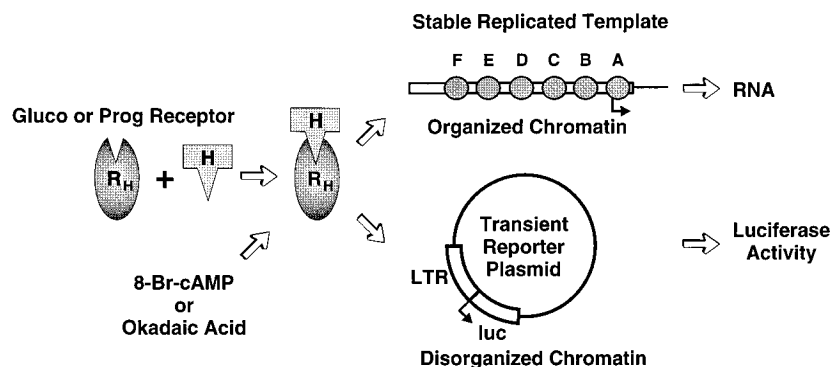


FIG. 1. Experimental design of the two-template model system. Cells containing stably replicating copies of the MMTV LTR driving various reporter genes are transiently transfected with a reporter plasmid containing the LTR driving the luciferase gene. The effects of hormones, 8-Br-cAMP, and okadaic acid on the two templates are measured in separate experiments by the assays shown. The two classes of templates are distinguished largely by the nucleoprotein structure they adopt in the cell, with the stably replicating template having an ordered structure and the transiently transfected template having a disorganized structure.

no change in nuclease accessibility or NF1 loading, but there appears to be a GR-induced binding of the initiation complex (6). Using this system, we can monitor the activities of these two classes of template in response to various effectors (Fig. 1), in this case, 8-Br-cAMP (an activator of the cAMP signalling system) and okadaic acid (an inhibitor of cellular phosphatases) as well as steroid hormones dexamethasone (Dex) and R5020, a synthetic progestin. Differences in the behaviors of these two classes of template allow us to identify the contribution of chromatin structure to the regulation of the MMTV promoter. An example of such differences has been recently described (60).

**Effects of 8-Br-cAMP and okadaic acid on the transient MMTV template.** Either 1470.2 cells or 3017.1 cells were transfected with pLTRluc and treated for 24 h with various combinations of effectors before activity was measured by luciferase assay. Both cell lines contain stably replicating MMTV templates consisting of the MMTV LTR driving the CAT gene in the context of BPV sequences (3). Cell line 1470.2 contains endogenous mouse GR, while 3017.1 cells, which were derived from 1470.2 cells (see Materials and Methods), express both mouse GR and chicken PR at roughly equivalent levels (60a). In agreement with the results of others (47, 49, 53), we find that both 8-Br-cAMP and okadaic acid can synergize with Dex in activating the transient template (Fig. 2A). On average, the fold induction over untreated controls is four times greater with Dex and 8-Br-cAMP than it is with Dex alone. Basal promoter activity is unaffected by 8-Br-cAMP alone, whereas okadaic acid alone increases it almost threefold. Figure 2B shows that the synergistic effect is not limited to the GR. In 3017.1 cells both 8-Br-cAMP and okadaic acid synergize with R5020, a synthetic progestin, in activating pLTRluc through the PR, although the magnitude of the synergistic effect is much less pronounced than that with activated GR. The effects of both 8-Br-cAMP and okadaic acid on basal activity are small (1.5- to 2-fold).

The MMTV LTR consists of multiple regulatory elements including an upstream enhancer with tissue-specific activity (36, 43, 45, 63), repressor regions (28, 34, 44, 46), and the proximal promoter with the hormone response elements, NF1 (15) and OTF1 (13, 41) sites, and the TATA box and initiator element (51). We used deletion analysis to determine which region of the LTR is responsible for the synergistic effect. Figure 2C shows that the synergistic effects of okadaic acid and 8-Br-cAMP are mediated largely through the proximal promoter. The p230 construct, which includes MMTV promoter

sequences to just beyond the glucocorticoid response elements, is poorly responsive to Dex (1.8-fold) but is significantly stimulated in the presence of Dex and either okadaic acid (6.4-fold) or 8-Br-cAMP (30.9-fold).

**Effects of 8-Br-cAMP and okadaic acid on the stable, replicated MMTV template.** Untransfected 1470.2 cells were treated with various effectors for 4 to 5 h, after which total RNA was extracted. Levels of MMTV mRNA were measured by S1 nuclease assay using an MMTV CAT-specific probe. Figure 3A shows that in 1470.2 cells Dex treatment causes a 38-fold increase in levels of MMTV mRNA. However, treatment with Dex and 8-Br-cAMP together (inset, lanes 3 and 4) results not in a synergistic effect, as observed with the transient template, but in an antagonistic effect. The inhibition of the Dex response is even greater if the cells are pretreated for 1 h with 8-Br-cAMP (inset, lanes 5 and 6), reducing the Dex-induced stimulation by over 75% (from 38- to 9-fold). There is also an 8-Br-cAMP-induced decrease in basal MMTV mRNA levels (approximately 30%). Interestingly, okadaic acid does not antagonize the Dex effect but causes increases in both basal (2-fold) and Dex-induced (69-fold) mRNA levels. To rule out effects of template copy number in the responses of the two templates to 8-Br-cAMP, this experiment was also carried out in 1470.2 cells transfected with MMTV templates, and the same results were observed: 8-Br-cAMP treatment led to repression of the replicating MMTV template and activation of the transient MMTV template (data not shown). It is therefore unlikely that the cause of the opposite responses to 8-Br-cAMP is the titration of limiting cellular factors.

The antagonistic activity of 8-Br-cAMP is also observed with activated PR. As shown in Fig. 3B the stimulatory effect of the PR, as well as basal activity, is inhibited by 8-Br-cAMP in 3017.1 cells in a dose-dependent manner. These results indicate that 8-Br-cAMP and okadaic acid are working through distinct mechanisms and that the antagonistic effect on the stable template is caused by a PKA-specific pathway which functions in the presence of activated GR or PR.

Treatment lengths of 4 to 5 h were used in these experiments (versus the 24-h treatments used for the transient MMTV template [Fig. 2]) because the Dex-induced increase in RNA generated from the stable, replicated MMTV templates is transient, peaking at 4 h and declining about 75% by 24 h (5). However, even after 24 h of treatment, 8-Br-cAMP is still able to cause a repression in Dex-induced MMTV RNA generated from the stable, replicating template. By the same token, after

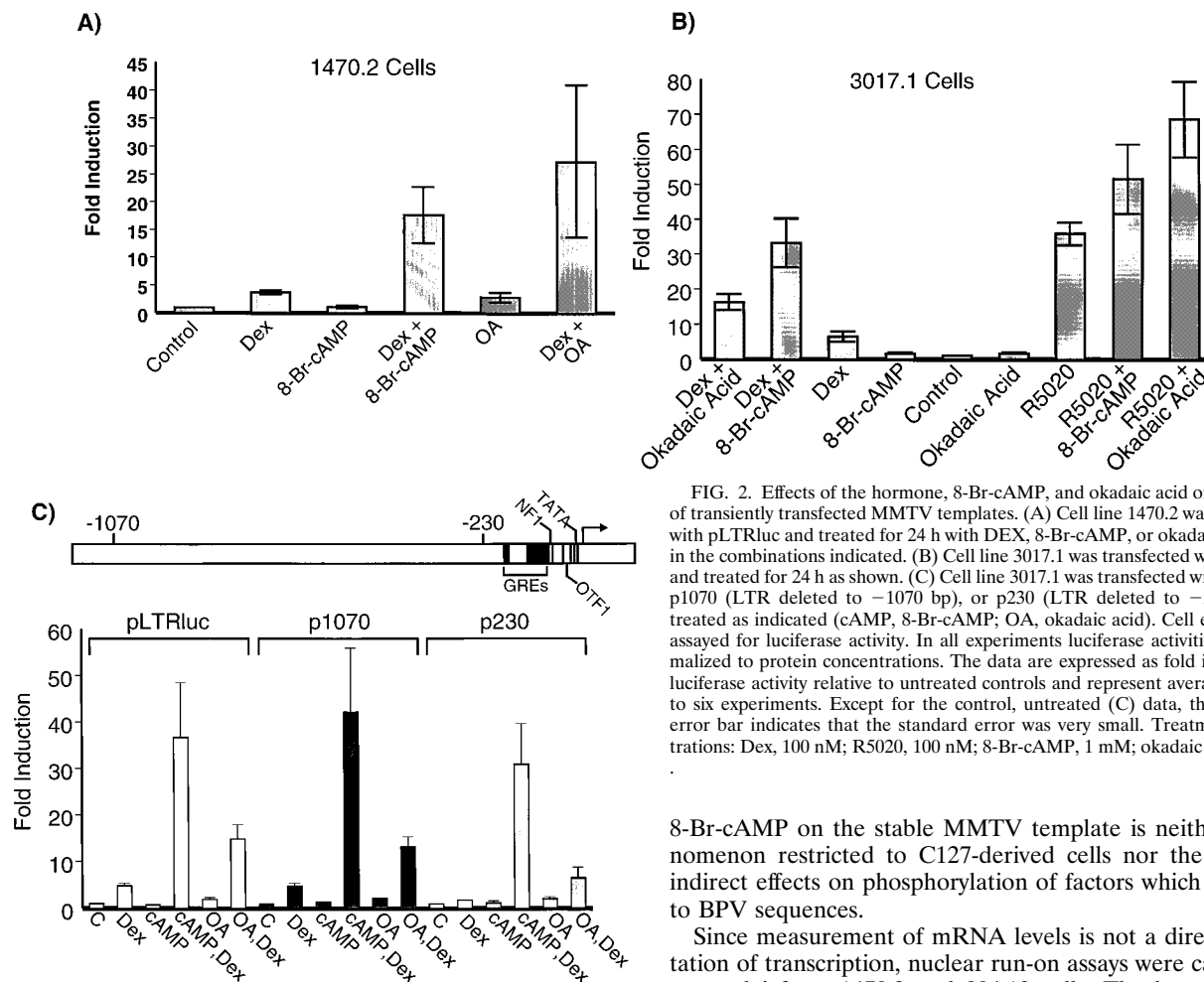


FIG. 2. Effects of the hormone, 8-Br-cAMP, and okadaic acid on the activity of transiently transfected MMTV templates. (A) Cell line 1470.2 was transfected with pLTRluc and treated for 24 h with DEX, 8-Br-cAMP, or okadaic acid (OA) in the combinations indicated. (B) Cell line 3017.1 was transfected with pLTRluc and treated for 24 h as shown. (C) Cell line 3017.1 was transfected with pLTRluc, p1070 (LTR deleted to  $-1070$  bp), or p230 (LTR deleted to  $-230$  bp) and treated as indicated (cAMP, 8-Br-cAMP; OA, okadaic acid). Cell extracts were assayed for luciferase activity. In all experiments luciferase activities were normalized to protein concentrations. The data are expressed as fold inductions in luciferase activity relative to untreated controls and represent averages of three to six experiments. Except for the control, untreated (C) data, the lack of an error bar indicates that the standard error was very small. Treatment concentrations: Dex, 100 nM; R5020, 100 nM; 8-Br-cAMP, 1 mM; okadaic acid, 50 nM.

4 h of treatment, the transient MMTV template is synergistically activated by 8-Br-cAMP and Dex (data not shown).

It is possible that the antagonism between the cAMP signalling pathway and steroid receptor activation is specific to these cell lines, which are both derived from C127 mouse mammary adenocarcinoma cells. In addition, the presence of BPV regulatory sequences around the MMTV LTR on the stable template may influence the response of the LTR to the hormone in the presence of 8-Br-cAMP. To address these issues, we carried out similar experiments with another cell line, 1505, which is derived from NIH 3T3 cells and contains a single copy of the MMTV LTR driving *H-ras* expression downstream of the Harvey sarcoma virus enhancer. The LTR has an ordered chromatin structure virtually identical to that in 1470.2 and 3017.1 cells, and the Nuc-B region undergoes a GR-induced structural transition (53a). As shown in Fig. 4, RNA generated from the stable template in 1505 cells is clearly reduced by 8-Br-cAMP treatment either in the presence or in the absence of the hormone. Also, in a fashion similar to that seen for 1470.2 cells, okadaic acid causes a reproducible, but small, increase in mRNA levels in the presence of Dex over those observed with Dex alone. Thus, the repression induced by activation of the cAMP signalling pathway is observed even in the absence of any BPV sequences. In addition, a transiently transfected MMTV CAT template which contains BPV sequences was not repressed by 8-Br-cAMP treatment (data not shown). These results imply that the antagonistic effect of

8-Br-cAMP on the stable MMTV template is neither a phenomenon restricted to C127-derived cells nor the result of indirect effects on phosphorylation of factors which may bind to BPV sequences.

Since measurement of mRNA levels is not a direct quantitation of transcription, nuclear run-on assays were carried out on nuclei from 1470.2 and 904.13 cells. The latter cell line contains 200 tandemly integrated copies of the BPV-MMTV LTR *ras* transcription unit, which has a nucleoprotein structure like that of the BPV-MMTV LTR CAT transcription units in 1470.2 cells. Figure 5 shows that treatment with 8-Br-cAMP and Dex reduces the amount of transcription from the stable MMTV template compared with that observed with Dex alone. In 1470.2 cells (Fig. 5B) hormone-induced transcription is reduced by 70%, while in 904.13 cells (Fig. 5A) it is reduced by 50%. Basal transcription is also significantly affected by 8-Br-cAMP treatment (reduced 70% in 904.13 cells and 50% in 1470.2 cells). Transcription from the actin promoter is slightly affected by 8-Br-cAMP treatment in both cell lines. The involvement of basal MMTV transcription in the 8-Br-cAMP effect implies that the mechanism by which the repression occurs is independent of GR or PR. Activation of these receptors only partially overcomes the inhibitory effect of the cAMP signalling pathway.

**Effects of 8-Br-cAMP on the nucleoprotein structure of the stable MMTV template.** To investigate the mechanism by which activation of the cAMP signalling pathway can lead to repression of the stable MMTV template, we carried out *in vivo* assays to monitor receptor-induced nucleoprotein alterations and transcription factor binding. A hallmark of the structural transition is a hormone-dependent increase in *SacI* endonuclease cleavage in the Nuc-B region of the stable MMTV template (4, 6). We digested nuclei from both 1470.2 and 904.13 cells with *SacI* after the same treatments as those used in the nuclear run-on assays. Figure 6 shows that Dex

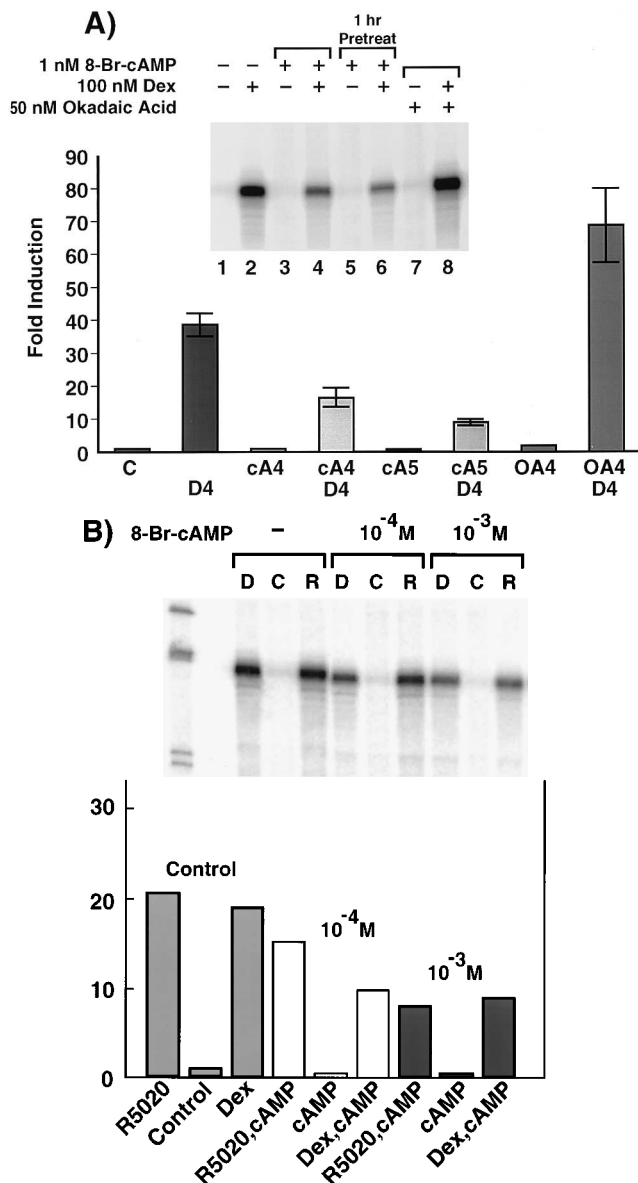


FIG. 3. Effects of the hormone 8-Br-cAMP and okadaic acid (OA) on levels of RNA derived from the stable MMTV template. (A) Cell line 1470.2 was treated as indicated. All treatments lasted 4 h except when cells were pretreated for 1 h with 8-Br-cAMP (cA) before the addition of Dex (D) (inset, lanes 5 and 6). Concentrations of the various reagents are identical to those in Fig. 2. The graphic data are expressed as the fold induction in mRNA relative to untreated, control (C) cultures and represent a summary of three experiments. Except for the control data, the lack of an error bar indicates a very small standard error. The inset shows a representative experiment in which RNA was subjected to S1 analysis. (B) Cell line 3017.1 was treated as indicated. All treatments lasted 4 h. The concentration of 8-Br-cAMP (cAMP) used is indicated; Dex (D) and R5020 (R) were used at 100 nM. The bar graph is a quantitative representation of the bands from the inset expressed as fold induction relative to the control, untreated sample. RNA levels were determined by S1 nuclease assay.

treatment induces *SacI* cleavage at the promoter in both cell lines. However, 8-Br-cAMP treatment has little effect on *SacI* cleavage in either cell line. Cleavage by *SacI* of the promoter in 1470.2 cells (Fig. 6A and B) is slightly reduced in the presence of both 8-Br-cAMP and Dex compared with that observed with Dex alone but does not reflect the dramatic drop in transcription (Fig. 5B). In addition, basal transcription was significantly

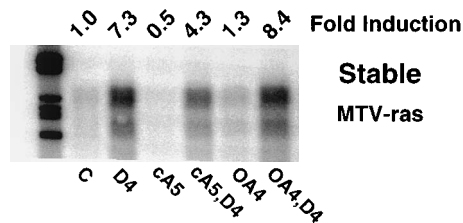


FIG. 4. Effects of the hormone 8-Br-cAMP and okadaic acid on the activity of a non-BPV-containing stable template in 1505 cells. Cell line 1505 was treated as indicated with Dex for 4 h (D4), 8-Br-cAMP for 5 h (cA5), and okadaic acid for 4 h (OA4). Lane C, untreated sample. RNA was subjected to S1 nuclease assay. The fold inductions relative to control, untreated cells are indicated. Concentrations of effectors are identical to those in Fig. 2. The pattern of inductions was reproducible in separate experiments.

reduced by 8-Br-cAMP, but under the same conditions cleavage of the promoter is not significantly changed in either 904.13 cells (Fig. 6C) or 1470.2 cells. Therefore, *SacI* cleavage of the stable MMTV template is largely unaffected by activation of the 8-Br-cAMP pathway.

Another characteristic of hormone-induced activation of the stable MMTV template is binding of NF1 (20), which is thought to be largely excluded from the template because of a closed chromatin conformation (4, 52). Activated receptor induces a transition to an open conformation, thereby allowing NF1 to bind. We carried out an exonuclease footprinting assay with 904.13 and 1470.2 nuclei, which were treated as described in the legend to Fig. 5. Figure 7 shows the well-characterized Dex-dependent binding of NF1 to its site in the promoter. However, treatment with Dex and 8-Br-cAMP together does not result in a reduction in NF1 binding, which appears unchanged even though transcription is greatly reduced. In these experiments we also observe a hormone-dependent exonuclease block downstream of the NF1 site at -50 bp, very close to the 5' edge of the proximal OTF consensus element in the promoter. Its response to 8-Br-cAMP and Dex treatment appears to be different in the two cell lines (Fig. 7). However, we have not found its behavior to be consistent between experiments. It is difficult to evaluate the binding of OTF1 in this type of experiment because the exonuclease must penetrate through the NF1 site (to which NF1 can be tightly bound) to proceed to the OTF1 sites. We are currently developing methods to assay its binding directly in vivo.

Since hormone-induced changes in the Nuc-B region are unaffected by 8-Br-cAMP treatment, we conclude that the receptor is able to bind the stable template when activated in the presence of 8-Br-cAMP and induce the characteristic structural changes in the Nuc-B region, including the increased binding of NF1. The results imply that the inhibitory effect is mediated through the basal transcription machinery or, possibly, the transcription elongation process, since the run-on transcription assay we have used does not distinguish between transcriptional initiation and elongation. To examine binding of factors to the TATA box region of the stable MMTV template, we carried out an exonuclease assay in which the entry point for the exonuclease was downstream of the transcription initiation site. The results are shown in Fig. 8 for 904.13 cells. (1470.2 cells do not have an appropriate restriction site downstream of the promoter to use as an entry point for the exonuclease.) Figure 8A (lane 2) shows that Dex treatment induces an exonuclease block at -17 bp relative to the start site. The proximity of this boundary to the TATA box indicates that it is associated with the binding of TFIID. A similar boundary has been described previously for the MMTV promoter (6, 35).

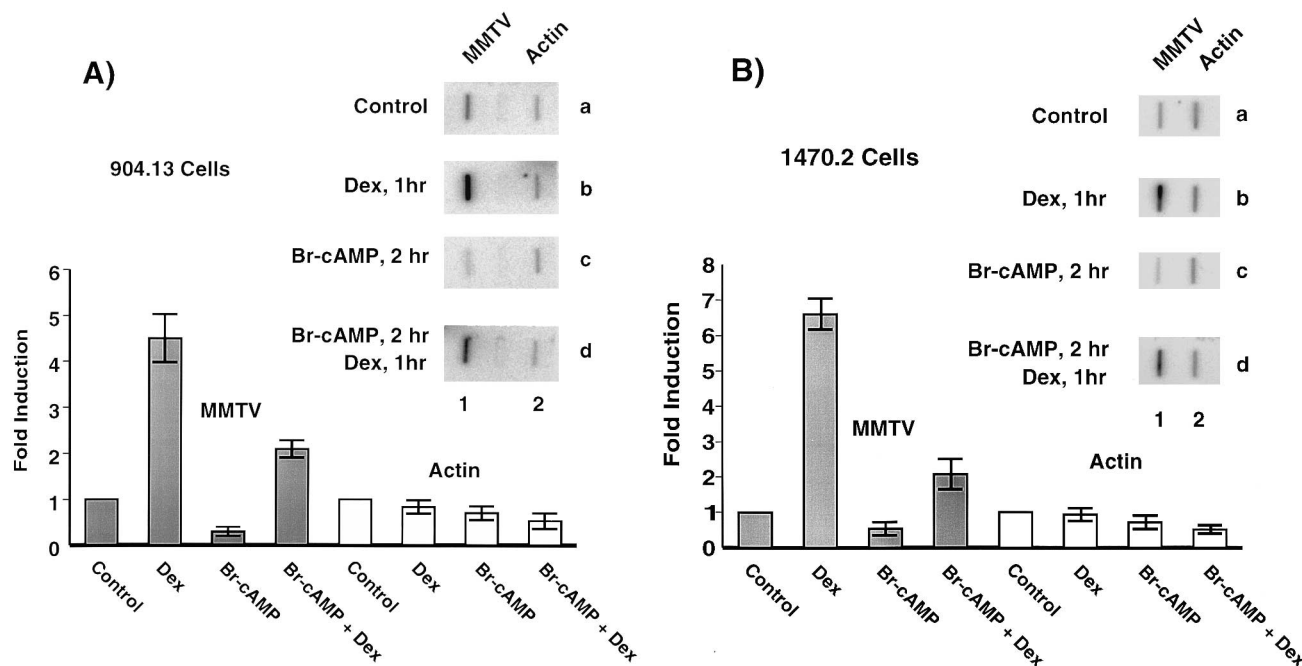


FIG. 5. Effects of the hormone 8-Br-cAMP on basal and hormone-induced run-on transcriptions from the stable MMTV template. Either 904.13 cells (A) or 1470.2 cells (B) were left untreated (control) (a) or were treated with Dex for 1 h (b), 8-Br-cAMP for 2 h (c), or Dex for 1 h and 8-Br-cAMP for 2 h such that Dex was added 1 h after 8-Br-cAMP (d). Run-on transcription was carried out on nuclei isolated from treated cells. Labelled RNA was hybridized to slot blots on which either MMTV, actin, or pUC18 (data not shown) had been immobilized. Quantitation was performed as described for Fig. 3, and the data from three separate experiments are presented graphically for MMTV and actin.

This block is also seen in lane 4, which contains DNA from nuclei treated with both Dex and 8-Br-cAMP. However, its intensity is reduced, implying that less TFIID is stably associated with the stable template under these conditions. In addition, a block at  $-38$  bp, which is just downstream of one of the OTF binding sites, shows a similar pattern of intensities. The reduction in both the TFIID and the OTF1 blocks upon 8-Br-cAMP and Dex treatment is very reproducible. Figures 8B and C represent the bracketed region of Fig. 8A from two additional experiments with 904.13 cells, in which Dex-induced transcription is reduced 50% (Fig. 5A) in the presence of 8-Br-cAMP. Unfortunately, this assay is not sensitive enough to detect TFIID and OTF1 binding in the untreated and 8-Br-cAMP-treated samples. However, these results lead us to conclude that the inhibition of MMTV transcription by activation of the cAMP signalling pathway is a result of impaired transcriptional initiation and, most likely, not an elongation effect.

## DISCUSSION

Activation of the cAMP signalling pathway or inhibition of cellular phosphatase activity can synergistically stimulate transcription from the MMTV promoter in the presence of glucocorticoids or progestins (10, 22, 47, 53, 57). We confirm that the response of transiently introduced MMTV reporter cassettes to GR and PR is cooperatively enhanced by cAMP. For replicated MMTV chromatin, however, activation of the cAMP signalling pathway represses the transcriptional activity of the promoter and inhibits its response to steroids. We infer that some feature unique to the integrated MMTV template is responsible for the differential response to activation of the PKA phosphorylation cascade.

Replicated MMTV chromatin is known to adopt a highly organized structure with positioned nucleosomes (54). The

promoter is relatively inaccessible to nucleases and transcription factors in the absence of a hormone. Activation by GR leads to a structural transition in the proximal promoter region characterized by loss of H1 (12), increased accessibility of nucleases (4, 54), and apparent loading of transcription factors (4, 20, 26). In contrast, in the same cells, transiently transfected MMTV templates are constitutively bound by NF1 and OTF1 (6, 35), while TFIID appears to associate with the TATA box in a hormone-dependent fashion (35). Transient templates also fail to adopt the positioned nucleosome array (6) but may have nucleosomes deposited in a disorganized fashion. These observations led us to propose that the structural transition in replicated chromatin is mechanically implicated in promoter activation (6, 27). Implicit in this model is the suggestion that interaction of the soluble transcription system with structured nucleoprotein templates is more complex than that with disorganized DNA templates and may involve components whose activity is not detected on disorganized templates. Functional differences between such templates have been described (16, 17, 35, 60), and the studies presented here provide another example of the differential activation potential of repressed, replicated templates compared with disorganized, transient templates.

We find that 8-Br-cAMP treatment represses basal transcription of replicated templates up to 70% and is antagonistic to the activation of transcription by ligand-associated GR and PR. This finding is remarkable in that it occurs in cells in which transient MMTV templates are activated by 8-Br-cAMP and Dex to a level four times greater than the level observed with Dex alone (Fig. 2A and B). The repressive effects of 8-Br-cAMP on the stable template are not due to the presence of flanking BPV sequences. Both basal expression and hormone-induced expression from an integrated MMTV template which does not contain BPV sequences were repressed by 8-Br-

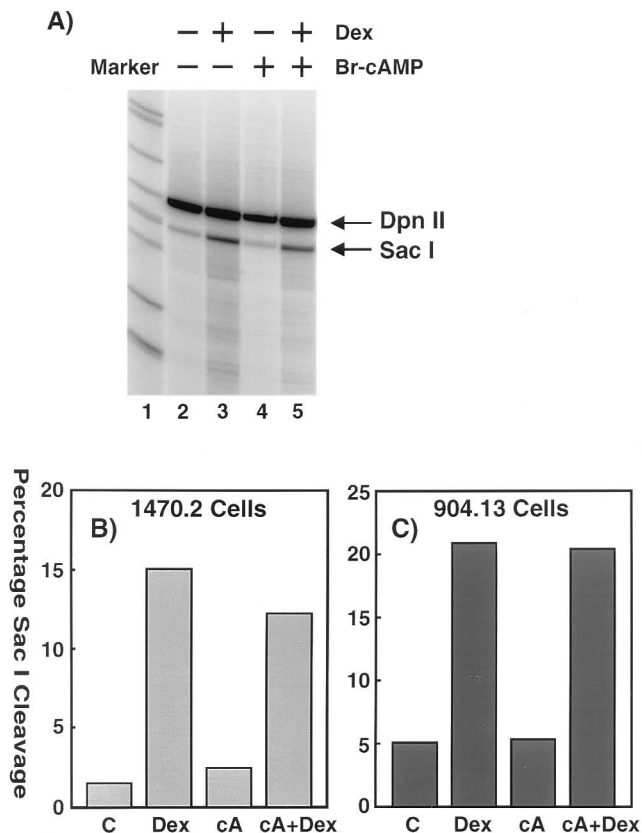


FIG. 6. Effects of Dex and 8-Br-cAMP (cA) on accessibility of the stable MMTV template to *SacI*. Either 904.13 cells or 1470.2 cells were treated as described for Fig. 5. Isolated nuclei were digested with *SacI*. DNA was purified and subjected to secondary digestion with *DpnII*. (A) Representative experiment with 1470.2 cells; (B) graphic representation of the data from panel A; (C) graphic representation of the same experiment carried out with 904.13 cells. Fractional *SacI* cleavage was calculated as a ratio of *SacI* cleavage to total cleavage by both *DpnII* and *SacI*.

cAMP (Fig. 4). In addition, neither basal activity nor hormone-induced activity of a transiently transfected BPV-MMTV CAT construct was repressed by 8-Br-cAMP treatment (data not shown).

Repression of the hormone-induced stable MMTV template may be mediated through loading of the basal preinitiation complex. Neither the hormone-dependent increase in *SacI* access nor NF1 binding is significantly affected by the presence of 8-Br-cAMP. Therefore, even when the cAMP signalling system is activated, the hormone-receptor complex can associate with the template and induce the structural transition associated with the increased transcriptional response. However, hormone-induced binding of the TFIID complex and OTF1 is reproducibly inhibited. The run-on transcription assays clearly indicate that the 8-Br-cAMP-induced repression of the stable MMTV template results primarily from inhibition of basal transcription. Although we cannot detect it, the transcriptionally active fraction of stable templates in the basal state must bind TFIID. Since basal transcription is clearly repressed by 8-Br-cAMP treatment, either the activity of the basal transcription machinery (the initiation complex) or its association with the stable template is inhibited. We postulate that the latter is more likely since we have shown that in the presence of the hormone the association of TFIID with the stable template is decreased if the cAMP signalling system has been activated. If only the activity of the basal machinery were affected, we might expect to have observed that the hormone-induced binding of TFIID was unaffected by the presence of 8-Br-cAMP. We are currently developing methods to directly measure transcription factor binding on uninduced templates *in vivo*.

The mechanism by which 8-Br-cAMP and okadaic acid synergize with activated GR or PR on transfected MMTV templates has been argued to involve changes in phosphorylation of nonreceptor factors, such as coactivators, transcription factors, or members of the basal transcription machinery, which can cooperate with activated PR or GR to stimulate MMTV transcription (9, 47, 49). Total phosphorylation levels of either intact receptor or peptide fragments are unchanged by treatment with 8-Br-cAMP or okadaic acid at concentrations which cause synergism with steroid hormones (10, 47, 57). The tran-

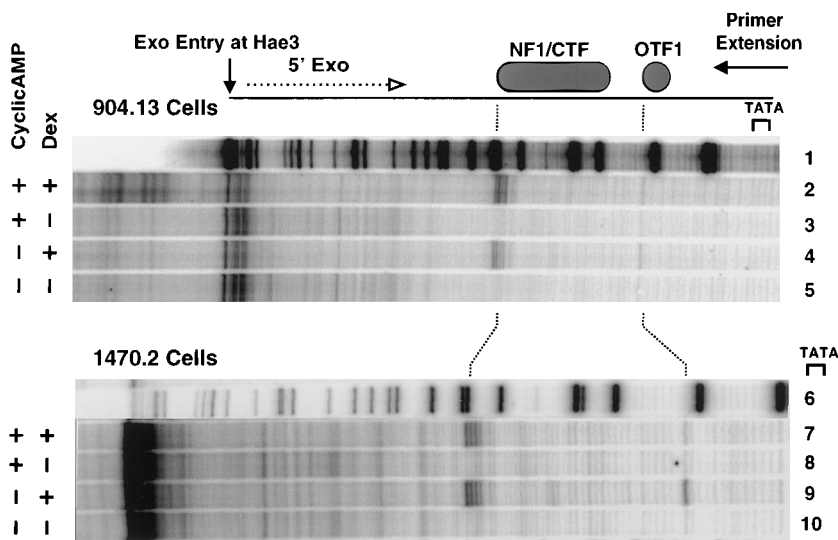


FIG. 7. Effects of DEX and 8-Br-cAMP on NF1 binding to the stable MMTV template. Either 904.13 cells (upper panel) or 1470.2 cells (lower panel) were treated as described for Fig. 5. Nuclei were digested with *HaeIII* and  $\lambda$  exonuclease (Exo), and DNA was processed as described in Materials and Methods.

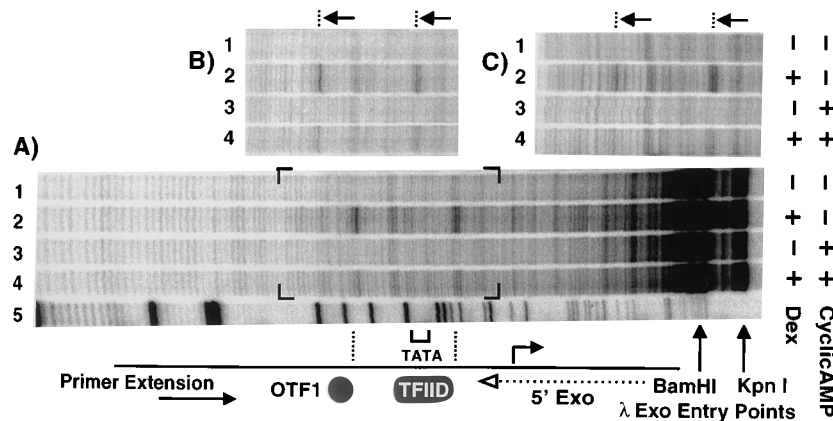


FIG. 8. Effects of Dex and 8-Br-cAMP on binding of TFIID to the stable MMTV template. 904.13 cells were treated as described for Fig. 5. Nuclei were digested with *Bam*HI, *Kpn*I, and  $\lambda$  exonuclease (Exo) (A) or with *Bam*HI and  $\lambda$  exonuclease (B and C). Panels A to C represent different experiments with 904.13 nuclei. DNA was processed as described in Materials and Methods.

scriptional synergism manifested on the transient template is induced by both 8-Br-cAMP and okadaic acid, but the repression of the stable template is seen only in the presence of 8-Br-cAMP, an observation which indicates that the response of the MMTV promoter to these effectors may involve two separate pathways. In fact, okadaic acid boosts both basal and hormone-induced levels of RNA generated by the stable template (Fig. 3A), and concomitant treatment with okadaic acid and 8-Br-cAMP does not reverse the repression seen with 8-Br-cAMP alone (data not shown).

Activation of the cAMP signalling pathway leads to more efficient interactions between transcription factors at the transiently transfected MMTV promoter, while it impairs interactions at the stable MMTV promoter. Thus, a separate target of the PKA-induced phosphorylation cascade may be involved in repression of the stable template. This activity would be dominant to the phosphorylated component(s) that enhances transient template activation but would not adversely affect transcription of the transient template. Candidate targets would include members of the basal machinery, upstream transcription factors such as OTF1, coactivators, repressors, histones, or phosphorylation-sensitive enzymes involved in the modification of these components. In fact, the DNA binding activity of OTF1 has been shown to be sensitive to phosphorylation (59). In addition, it can physically interact with TBP *in vivo* (66) and is important for basal promoter activity of integrated MMTV templates (14). Since its binding to the replicated MMTV template is diminished in the presence of 8-Br-cAMP and Dex, OTF1 recruitment is a potential candidate for mediating the differential effect.

Replicated MMTV templates acquire positioned nucleosomes and are repressed by some property of chromatin. In this environment, changes in the net charge of transcription factor domains caused by phosphorylation may lead to altered interactions with histones, which have highly charged N-terminal tail regions. Alternatively, these alterations may arise through phosphorylation of histones. Elegant studies with *Saccharomyces cerevisiae* have shown that histone tails, which are highly basic and contain sites for acetylation and phosphorylation (reviewed in reference 11), can play a role in transcriptional regulation, most likely through interactions with soluble transcription factors and/or repressors (24, 31, 33, 39, 56). Various effectors have been shown to induce changes in histone phosphorylation in mammalian cells (1, 19, 38, 40, 62). In fact, okadaic acid treatment of C3H10T1/2 cells leads to rapid

phosphorylation of H3 (38), while treatment of HL60 cells with vitamin D<sub>3</sub> leads to dephosphorylation of histones H3 and H2B (40). In any case, altered factor-histone interactions might be inhibitory to transcription, leading to a loss of communication between upstream factors or coactivators and the basal transcriptional machinery or a destabilized association with the template. These interactions may not take place at the transient MMTV template given its disorganized nucleoprotein structure, and therefore, it would not respond in an inhibitory manner.

Two previous studies concerning the effects of cAMP and hormones on MMTV transcription, carried out with integrated reporter constructs, reported synergistic activation in the presence of 8-Br-cAMP and steroids (10, 47), findings at variance with those reported here. These studies were both carried out with one cell type (T47D [human mammary adenocarcinoma]), in which the nucleoprotein structure of the integrated MMTV promoter was uncharacterized. It is possible that there are cell-type-specific components in the pathway leading to repression of the stably replicating template. Alternatively, the structure of the stably replicating MMTV template may be different in these T47D lines such that it is unaffected by this regulatory pathway. In support of this idea, we have observed that stably replicating MMTV templates in T47D cells can adopt a different, constitutively open nucleoprotein structure in the absence of the hormone (48). Thus, the MMTV template structure in these cells is similar to that of transient templates in that the promoter is not repressed by chromatin. Therefore, the structure which a replicating MMTV template adopts may be the determining factor in its response to cAMP signalling. The causes for these differences in structure are not presently understood.

Finally, the results described here also indicate that the hormone-dependent structural alterations in promoter chromatin can be dissociated from activated transcription. That is, the development of a complete hypersensitive transition still occurs when formation of the preinitiation complex is inhibited. This means that a productive interaction between a template-bound receptor and members of the proximal initiation complex is not necessary for the hormone-dependent chromatin structural transition and, furthermore, that the structural alteration is not the result of increased transcription. Similar conclusions have been reached by Fascher et al. for the chromatin transition induced at the PHO5 promoter in *S. cerevisiae* (25).



The most important observation of this study is that two types of the MMTV template having the same LTR sequence, but differing significantly in their nucleoprotein structure, can, in the same cells, have opposite responses to the activation of a cellular signalling pathway. This system highlights the role chromatin structure can play in the regulation of transcription *in vivo* and underlines the necessity of studying physiological transcriptional templates. In the living organism simultaneous activation of different signalling pathways is likely to be a regular event, so it is important to know how genes affected by crosstalk between those pathways will react in a natural setting. The study of transfected promoters is important for understanding the interactions and functions of soluble transcription factors, but the study of chromatin templates provides insight into the behavior of soluble factors in the more physiological context of ordered chromatin.

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