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Newly expressed progesterone receptor cannot activate stable, replicated mouse mammary tumor virus templates but acquires transactivation potential upon continuous expression

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ABSTRACT **During development and differentiation, the expression of transcription factors is regulated in a temporal fashion. Newly expressed transcription factors must interact productively with target genes organized in chromatin. Although the mechanisms governing factor binding to chromatin templates are not well understood, it is now clear that template access can be dramatically influenced by nucleoprotein structure. We have examined the ability of a well characterized transactivator, the progesterone receptor (PR), to activate the mouse mammary tumor virus (MMTV) promoter organized either in stable, replicating templates that have a highly ordered nucleosome structure or as transiently transfected DNA, which adopts a less-defined structure. If the PR is transiently expressed in cells harboring both replicated and transient MMTV reporter constructs, it cannot significantly activate the stable replicated MMTV template. In contrast, when PR cDNA is stably inserted into the same cells and constitutively expressed, it gains the ability to activate both chromosomal and transiently introduced templates. These results demonstrate that newly expressed PR is not competent to activate the MMTV template in its native nucleoprotein conformation but acquires this ability upon prolonged expression in replicating cells.**

In eukaryotic cells, genes are expressed from chromatin templates. Various studies have shown that nucleoprotein structure plays a role in transcriptional regulation by restricting the access of some factors to their binding sites while allowing that of others by mechanisms not yet understood (1–7). The mouse mammary tumor virus (MMTV) promoter exists in chromatin as a phased array of six nucleosomes (8). In this structural configuration, nuclear factor 1 (NF1) is excluded from its target site in the proximal promoter (1, 6, 9, 10). Binding of the activated glucocorticoid receptor (GR) to the promoter induces a chromatin remodeling event associated with the second nucleosome (Nuc-B) (8, 11). In addition, histone H1 is depleted from the promoter proximal region in a hormone-dependent manner (12). We proposed previously that this chromatin transition is directly and mechanistically involved in the binding of NF1 and subsequent formation of the transcription preinitiation complex (9, 10). Similar phenomena have been observed for the murine tyrosine aminotransferase (13–15) and yeast *pho5* (2, 16) genes.

The progesterone receptor (PR) and GR activate the MMTV promoter through the same target sequences as determined by transient transfections (17–20). During experiments designed to characterize the kinetics of steroid receptor interaction with MMTV chromatin, we observed that transiently expressed PR was apparently ineffective in activating a replicated MMTV template. To address this question directly, we have developed a general methodology to compare the activation potential of transcription factors on replicated and transient templates. A template expressing a cellular marker is cotransfected with expression and reporter plasmids of interest. After expression, cells are separated based on the transfected cellular marker, using fluorescence-activated cell sorting (FACS). Selected pools are enriched to the extent that 60–75% of cells contain transiently introduced DNA. This method allows us to compare directly the function of either transiently expressed, or endogenous, transcription factors on a cotransfected transient template or an endogenous, genomic template.

To examine the function of the PR on prolonged expression, we stably introduced the PR into cells to generate clonal lines that express both GR and PR constitutively. We find that when expressed transiently, the PR fails to significantly activate the stably replicated MMTV template, although it is active on a transiently introduced MMTV template. In contrast, when the PR is expressed constitutively from genomic copies of the cDNA, the receptor acquires the ability to activate both templates.

METHODS

Cell Lines. Cell lines 1505 and 3036.2 were grown in Dulbecco's modified Eagle's medium containing 10% charcoal-stripped serum. Cell line 1505 is derived from NIH 3T3 cells into which a single copy of a MMTV ras transcription unit was inserted (21). Cell line 3036.2 was derived from 1505 cells by stable transfection of the chicken PR expression vector pcPRO. Neomycin-resistance vector, pRSVneo, and pcPRO were cotransfected into 1505 cells. Colonies were selected by G418 (Geneticin, Life Technologies), expanded, and assayed for activation of both transient and stably replicating MMTV templates by R5020 treatment. Cell line 3036.2 was isolated from R5020-responsive cell pool 3036 by single cell cloning.

Transient Transfection Assays. For sorting, 1505 cells were transfected by calcium phosphate precipitation in 100-mm dishes with 8 μg each of pLTRluc [full length MMTV long terminal repeat (LTR)-driving luciferase], pcPRO, and pCH110 [Pharmacia; β-galactosidase (β-Gal) expression vector]. Two days after transfection, cells were treated with control medium, dexamethasone (Dex), or R5020 (both 0.1 μM). 3036.2 cells were also transfected by calcium phosphate

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Abbreviations: MMTV, mouse mammary tumor virus; PR, progesterone receptor; GR, glucocorticoid receptor; NF1, nuclear factor 1; FDG, fluorescein di-β-galactopyranoside; β-Gal, β-galactosidase; FACS, fluorescence-activated cell sorting; LTR, long terminal repeat.

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precipitation in six-well dishes with 1 μg of pLTRluc. Luciferase activities were determined and normalized to total protein.

FACS Methods. Nontreated and R5020-treated cells were briefly treated with trypsin and neutralized with trypsin inhibitor (Calbiochem). Cells from each group were pooled and treated according to specifications in the FluoReporter lacZ kit (Molecular Probes). Briefly, cells were washed with phosphate-buffered saline (PBS) and resuspended in staining medium (PBS/4% charcoal-stripped serum/10 mm Hepes, pH 7.2). After filtration through a nylon screen, the cells were mixed with an equal vol of 2 mM fluorescein di-β-galactopyranoside (FDG) and incubated at 37°C for 1 min. The cells were diluted immediately in 10 vol of ice-cold staining medium containing 15 μM propidium iodide and allowed to incubate on ice for 15 min, after which the β-Gal inhibitor phenethylthio β-D-galactopyranoside was added to a final concentration of 1 mM. Each set of cells was then sorted into two populations having low (β-Gal$^-$) or high (β-Gal$^+$) FDG fluorescence intensity in a Becton Dickinson FACStar Plus set in the three-drop enrichment sorting mode. High-purity sorting would not have allowed us to sort enough β-Gal$^+$ cells in a reasonable amount of time to yield enough RNA for analysis.

RNA Analysis. Total cellular RNA was isolated from cells as described (22). The probe for S1 nuclease analysis was made by multiple rounds of *Taq* polymerase extension from an antisense luciferase oligonucleotide using *Sst* I-digested pLTRluc (23) as a template. Extension was carried out in the presence of [α-^{32}P]dATP in a Perkin–Elmer/Cetus GeneAmp PCR system 9600 machine for 30 cycles. The antisense luciferase primer (+80 to +55 bp), 5′-CCTTTCTTTATGTTTTTGGCGTCTTC-3′, was synthesized on an Applied Biosystems model 392 DNA/RNA synthesizer. The full-length extension product was gel purified. After hybridization with RNA samples, S1 nuclease digestion was performed for 1 hr at room temperature. Digestion products were separated on an 8% denaturing urea gel, which was subjected to autoradiography. Primer-extension analysis was performed as described (10).

Ligand Binding Assays. Transfected 1505 cells (untreated) were sorted. Cytosols were made from the transfected population as well as from 3036.2 cells by Dounce homogenization in HEDM (10 mM Hepes, pH 7.3/1 mM EDTA/1 mM dithiothreitol/10 mM sodium molybdate), addition of glycerol to a final concentration of 10%, and centrifugation at 100,000 × *g*. Protein concentration was determined by the Bradford method. Two hundred micrograms of cytosolic protein was incubated with 75 nM [^{3}H]R5020 (New England Nuclear) in the presence or absence of a 500-fold excess of unlabeled R5020 for 90 min. Dextran-coated charcoal [4% Norit-A (ICN)/0.4% Dextran T-70 (Pharmacia)] was added. Samples were Vortex mixed and incubated on ice for 10 min. The charcoal was pelleted and the bound steroid in the supernatant was determined by liquid scintillation counting.

RESULTS

Experimental Design. Cell line 1505, which is derived from NIH 3T3 cells, does not express PR. This cell contains one integrated copy of the MMTV LTR fused to the Ha-v-*ras* oncogene. The LTR is organized as a phased array of nucleosomes and undergoes a structural transition upon glucocorticoid treatment similar to that previously reported for bovine papilloma virus-based episomes (8, 21). After introduction of the chicken PR by transient transfection, we compared the activity of the receptor on the genomic MMTV promoters and transiently cotransfected templates.

Since only 5% of the cells actually acquire transfected DNA (see Fig. 2 *A* and *B*), a direct comparison of activity on the stable and transient templates is technically difficult. Cells that take up DNA will contain both the transient reporter template and the transiently expressed PR; in contrast, all cells in the population contain the stable template, but only the small transfected subset expresses the PR.

To more accurately compare the activity of the PR on the two templates, we devised an experimental approach to obtain a cell population enriched in cells transfected with exogenous DNA (and therefore expressing PR). FACS analysis has been used to enrich cells stably transfected with the β-Gal gene driven by various promoters (24–28). We adapted this procedure for use with cells transiently transfected with a β-Gal expression vector (pCH110), as well as pLTRluc, containing the full-length MMTV LTR-driving luciferase, and the PR expression vector, as shown in Fig. 1. After transfection, the cells are divided into two groups; one was left untreated, and the other was treated with R5020 (a synthetic ligand for PR) for 4 hr. After harvesting, cells from each group were incubated with a β-Gal substrate, FDG, which releases fluorescein upon cleavage. Cells that take up exogenous DNA, and therefore express β-Gal (β-Gal$^+$), were separated from the untransfected cells (β-Gal$^-$) by enrichment sorting in a FACS. The β-Gal$^+$ populations obtained in separate sorting experiments represent at least a 10-fold enrichment in transfected cells over the unsorted cell populations, permitting a more rigorous comparison of the function of the transiently expressed PR on both stably replicating and transiently transfected MMTV templates.

Fluorescence profiles from a representative sorting experiment are shown in Fig. 2. Fig. 2 *A* and *B* shows the fluorescence profiles of unsorted control and R5020-treated cell populations, respectively. Most of the cells (≈95%) manifested low propidium iodide fluorescence and therefore represented viable cells. The large mass of cells of intermediate FDG fluorescence is the β-Gal$^-$ population; the scattered population of cells at higher FDG fluorescence represents β-Gal-expressing cells, ≈5% of the viable population. Fig. 2 *C* and *D* represents histograms of the control and R5020-treated β-Gal$^-$ populations that were analyzed after sorting. These groups consist of an essentially pure population of cells having basal fluorescence intensity. In contrast, the control and R5020-treated β-Gal$^+$ populations (Fig. 2 *E* and *F*) consist of two sets of cells. The peaks of higher FDG fluorescence intensity represent β-Gal-expressing cells, which make up 74% of the cells in the control and 65% in the R5020-treated β-Gal$^+$ populations.

PR Function When Transiently Expressed. After sorting, RNA was isolated from the various cell populations, as well as from unsorted cells, and subjected to S1 nuclease analysis with a probe designed to detect transcripts from both the

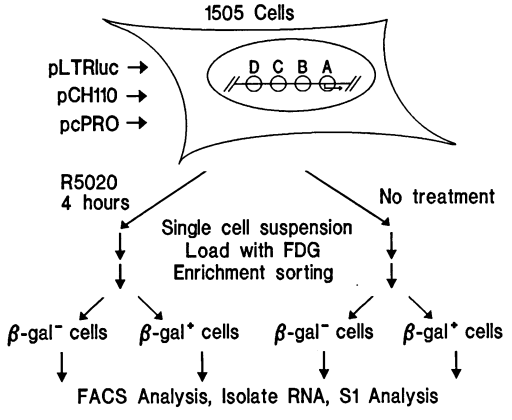

FIG. 1. Experimental strategy to sort 1505 cells into transfected and nontransfected populations and analyze RNA generated from two MMTV templates.

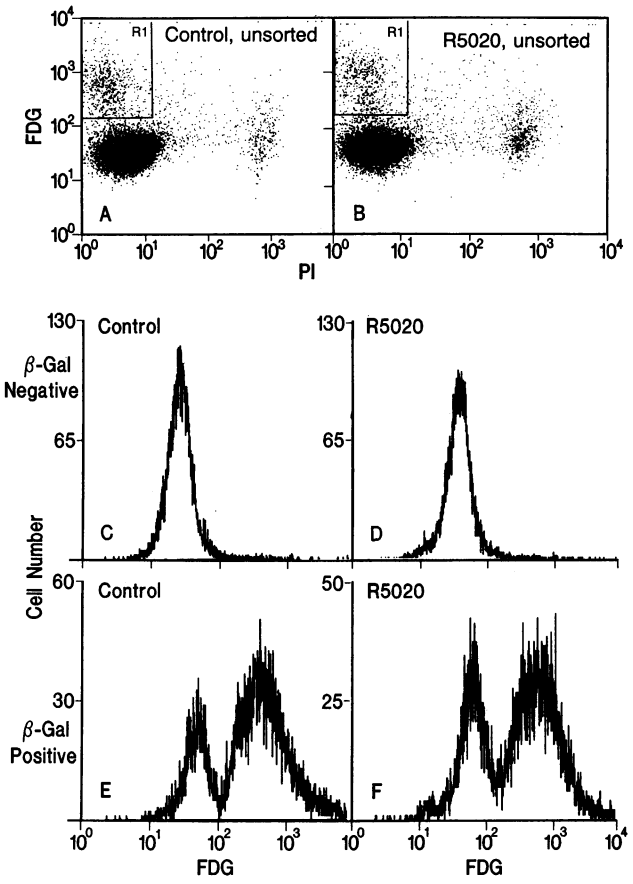

FIG. 2. Fluorescence-activated sorting of transfected cells. (*A* and *B*) Fluorescence profiles of unsorted control (*A*) and R5020-treated (*B*) cell populations. FDG (fluorescein) and propidium iodide (PI) fluorescence intensities were measured in the unsorted cell populations and used to set sorting gates for the β-Gal$^-$ and β-Gal$^+$ populations; gate settings for the latter are indicated by the small box (upper left). Approximately 5% of the each cell population is contained in the gated area. The sorting gate for the β-Gal$^-$ population is not shown but included a portion of the large mass of cells having lower FDG fluorescence intensity. (*C–F*) FDG fluorescence profiles of sorted cell populations. After sorting, aliquots of the four sorted populations were analyzed in the FACS to determine cell composition. (*C* and *D*) β-Gal$^-$ populations. (*E* and *F*) β-Gal$^+$ populations. (*C* and *E*) Populations sorted from control cells. (*D* and *F*) Populations sorted from R5020-treated cells.

transiently transfected and the stably replicating MMTV templates (Fig. 3*A*). RNA analysis from a representative experiment (Fig. 3*B*) shows that the transcript from the transient template is marginally detected in the control and R5020-treated β-Gal$^-$ populations (lanes 4 and 5), while it is clearly seen in the β-Gal$^+$ populations (lanes 6 and 7), indicating that the transfected DNAs were successfully concentrated in the β-Gal$^+$ population. Dex treatment induces transcription from both the transient and stable MMTV templates in the unsorted cells (lanes 1 and 2), as expected. In the β-Gal$^+$ populations (lanes 6 and 7), we observe induction of the transient transcript by R5020 (lane 7), but this is not accompanied by a significant induction of the transcript from the stable MMTV template. To determine whether this difference between PR and GR action has a kinetic basis, we performed the same experiment except that R5020 treatment was for 24 hr. The results (Fig. 3*C*) show that the stable template is not significantly induced even with longer R5020 treatment.

Table 1 shows a summary of the results from three separate sorting experiments. Dex treatment causes a 2- to 3-fold

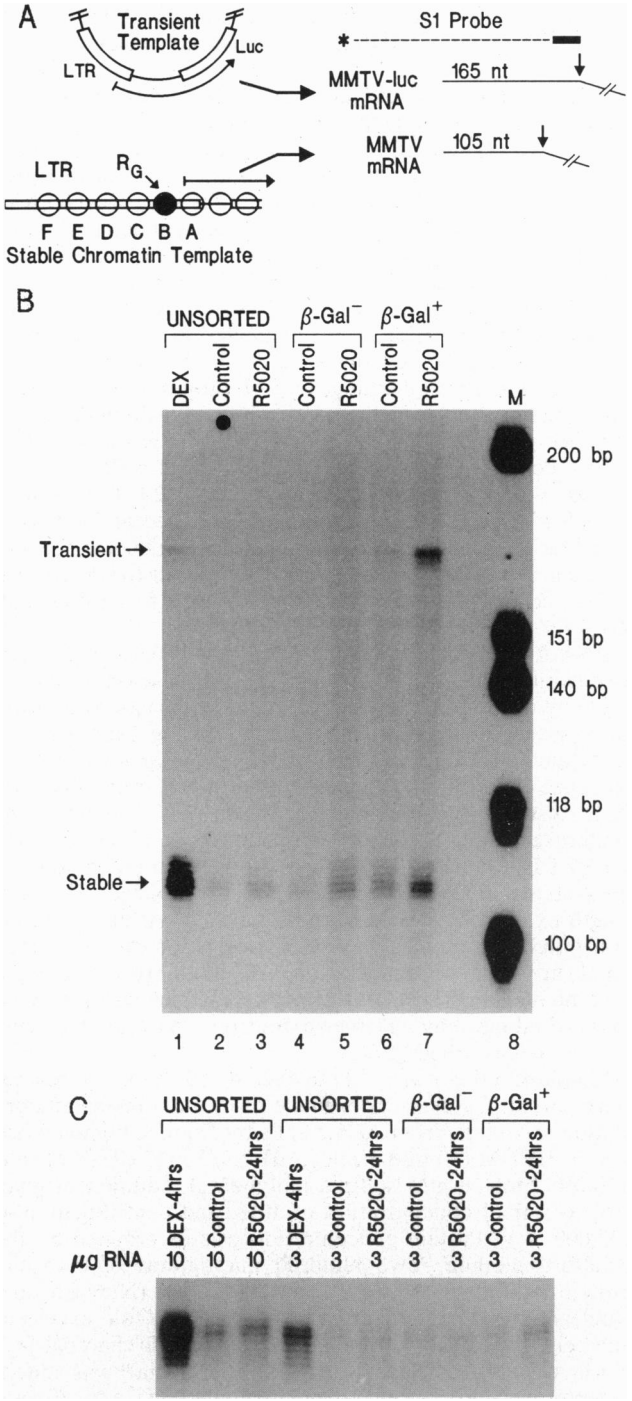

FIG. 3. Differential activation by R5020 of two MMTV templates in sorted cells enriched in transfected DNA. (*A*) A probe for S1 nuclease analysis was prepared that will differentiate between transcripts from the two MMTV templates. (*B*) Total RNA from unsorted and sorted cell populations (4 μg of each sample) was subjected to S1 nuclease analysis. Lanes 1–3, unsorted cells transfected with the same DNAs as the sorted cells shown in lanes 4–7. Lane 1 contains RNA from Dex-treated (0.1 μM; 4 hr) cells; lanes 2, 4, and 6 contain RNA from untreated, control cells; lanes 3, 5, and 7 contain RNA from cells treated with 0.1 μM R5020 for 4 hr. Lane 8 contains *Hin*fI-digested OX174 DNA. (*C*) Analysis of RNA from sorted and unsorted cell populations in which R5020 treatment was for 24 hr.

greater induction of the transient template at 4 hr relative to R5020 (comparing induced levels of transient mRNA in unsorted cells). However, while Dex treatment causes an ≈10-fold induction of RNA derived from the stable MMTV

template, R5020 treatment (4 hr) yields only a slight 1.6-fold induction in the β-Gal$^+$ populations. In the same cell population, the transient template is induced an average of 5-fold by R5020. The small, R5020-induced increase in RNA from the stable templates may represent either a weak ability of the PR to activate the template in its nucleoprotein conformation or the existence of a small subset of MMTV chromatin templates that, like the transient template, have a structure amenable to PR-induced activation. This state might be achieved during replication when the nucleoprotein structure is reassembled.

PR Function When Expressed Constitutively. It has been reported that the PR can activate transcription from integrated, replicating copies of the MMTV LTR, although the chromatin structure of the LTR in these studies was not characterized (19, 20). We therefore determined whether the PR, when expressed constitutively, could activate the LTR in cell lines with a defined and reproducible chromatin configuration.

Clonal isolates of 1505 cells were established with the chicken PR cDNA stably integrated in the genome. Cell line 3036.2, a representative example, expresses PR in addition to GR. Expression from the stable template was examined by primer-extension analysis on RNA isolated from cells treated 4 hr with Dex or R5020, as well as from untreated cells. Expression of luciferase from the transient template was carried out on 3036.2 cells transfected with pLTRluc and similarly treated. The stably replicating (Fig. 4*A*) MMTV template is induced by either Dex or R5020 in 3036.2 cells but only by Dex in 1505 cells. The transient MMTV template (Fig. 4*B*) is induced by both Dex and R5020 in 3036.2 cells.

Levels of PR Expression in Transiently and Stably Transfected Cells. One could postulate that the levels of PR required to fully activate the stably replicating MMTV template are higher than those required to activate the transient MMTV template and that there is not enough PR expressed in the transiently transfected 1505 cells to activate the stably replicating template. To determine the level of PR expression in transiently and stably transfected cells, we carried out single-point hormone binding analysis on cytosols from 3036.2 cells and the sorted, transfected population of 1505 cells. The results of two different experiments are shown in Table 2. The average expression levels of PR in transiently transfected 1505 cells are equal to, or greater than, those in 3036.2 cells. It is therefore unlikely that insufficient PR expression levels are responsible for the differential effect of PR on the stably replicated and transient MMTV templates.

Table 1. GR and PR response on transient and stable templates

	Exp. 1		Exp. 2		Exp. 3	
Template	Units	Ind	Units	Ind	Units	Ind
Stable						
Unsorted						
Dex	8972	10.0	4712	10.9	7262	7.2
Control	893		432		1015	
R5020	1573	1.8	650	1.5	791	0.8
β-Gal$^-$						
Control	870		ND		660	
R5020	1308	1.5	ND		644	1.0
β-Gal$^+$						
Control	1374		817		1200	
R5020	2236	1.6	1288	1.6	1827	1.6
Transient						
Unsorted						
Dex	314	—	261	—	1022	—
R5020	147	—	89	—	633	—
β-Gal$^+$						
Control	270		237		201	
R5020	1373	5.1	939	4.0	1273	6.3

Cells were transfected, treated (all R5020 treatments were 4 hr), and sorted as described. Autoradiographs from subsequent RNA analyses were subjected to densitometric scanning with a densitometer (Molecular Dynamics). The intensities of RNA/DNA S1 nuclease-resistant hybrids were quantified by using arbitrary units. Control levels of mRNA generated by the transient template in unsorted cells were too low to be accurately quantitated and are therefore not included. Ind, induction; ND, not determinable due to unequal backgrounds.

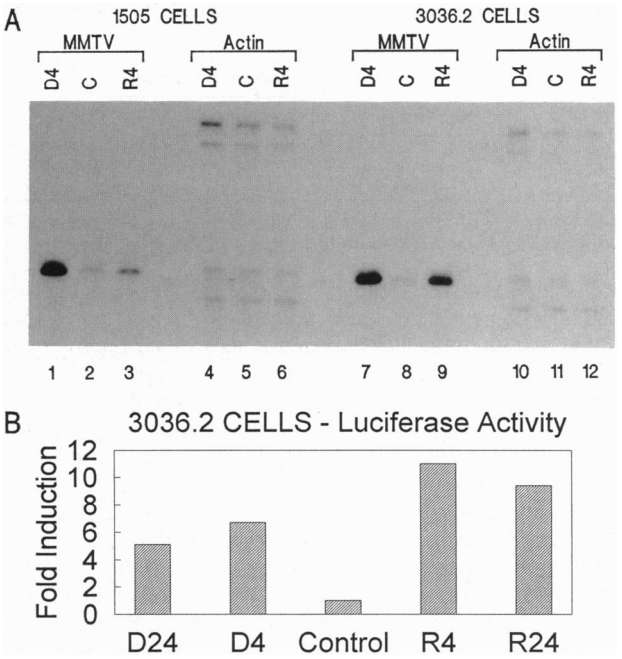

FIG. 4. PR is able to activate both MMTV templates when expressed constitutively. (*A*) Primer-extension analysis of RNA isolated from 1505 (lanes 1–6) and 3036.2 cells (lanes 7–12) that were treated either with Dex (lanes 1, 4, 7, and 10) or R5020 (lanes 2, 5, 8, and 11) or left untreated (lanes 3, 6, 9, and 12). (*B*) Luciferase analysis of 3036.2 cells transfected with pLTRluc (1 μg) and either untreated (Control) or treated with Dex (D) or R5020 (R) for 4 or 24 hrs as indicated.

DISCUSSION

We have shown that transiently expressed PR does not significantly activate a MMTV template with an ordered chromatin structure. In contrast, it does activate a transiently introduced template with a disorganized nucleoprotein structure. These results imply that there is an additional requirement for activation by the PR when the MMTV template is in a highly structured nucleoprotein conformation. After long-term constitutive expression of the PR, this requirement is fulfilled, and the stable, replicated MMTV template is efficiently activated by progestin treatment. This requirement is not due to expression levels of the PR, nor is it fulfilled by longer progestin treatment in transiently transfected cells.

We have previously shown that the stably replicating MMTV template is in a repressed conformation (1, 9, 10). Some members of the transcription initiation complex, NF1 in particular, are blocked from binding by the presence of a nucleosome over the promoter. Glucocorticoid treatment results in a chromatin remodeling event that allows access of NF1 and recruitment of other factors to the template to activate transcription (8, 10, 11). Histone H1 is also depleted from the proximal promoter region during this remodeling event (12).

Two classes of models can be advanced to explain the differential interactions of the transiently expressed PR with

Table 2. Ligand binding analysis in 3036.2 cells and the transfected population of 1505 cells

Cell line	Specific binding* Exp. 1	Exp. 2
3036.2	2299	1690
1505 (transfected)	4332	1610

*Levels of specific [^{3}H]R5020 binding are expressed as cpm per 100 μg of total cytosolic protein.

stable and transient MMTV templates. The first class includes mechanisms related to processing of receptors or potential cofactors. The transiently expressed receptor may be incompletely processed with regard to posttranslational modifications required for productive chromatin interaction, or cofactors required for chromatin activation are induced by the receptor itself and thus absent in transiently transfected cells. In this line of argument, when the PR is stably integrated and expressed over many cell generations, processing steps (or the induction of cofactors) needed for effective nucleoprotein interactions are completed and the receptor is effective in chromatin activation. Alternatively, the PR may not be able to compete efficiently with the GR for common factors necessary for activation of ordered chromatin templates when expressed for a short period. Candidates for such activities would include mammalian equivalents of the SWI-1, -2, -3 factors (29, 30), as well as modification enzymes such as protein kinases. Both possibilities imply the existence of processing events or cofactors that are uniquely required for activation of the ordered nucleoprotein template.

A second group of models would suggest that the nucleoprotein conformation of the stably replicating template is incompatible with some effect of PR action, such as secondary loading of other transcription factors specific to the PR; this would not be observed with the transient template given its generally more accessible structure (10). In cells in which the PR is constitutively expressed, we hypothesize that the structure of stably replicating MMTV templates may be modified in such a way that the promoter is now able to respond to both receptors. During transient transfection assays, 1505 cells undergo approximately one round of replication, since their growth is slowed considerably by calcium phosphate precipitation. By the time the stable cell lines are assayed for the presence of PR they have undergone multiple rounds of replication. Although the detailed steps in receptor DNA binding and transactivation are still not well understood, several studies have shown that the estrogen receptor (ER) and PR are localized to the nucleus in the uninduced state (31–33). In fact, there are reports that the ER is associated with its binding site in the absence of hormone (34, 35). We therefore suggest that the PR may associate with its site during replication when nucleoprotein constraints are temporarily removed. In this fashion it could alter the nucleoprotein conformation of the stable template in such a way that the promoter is then poised to respond to progestins.

There are several known examples of genes that undergo chromatin transitions during differentiation. With our increased understanding of nucleoprotein structure, it is becoming possible to address the role that these remodeling events play in modifying transcription factor access. The system described in this report allows us the opportunity to study how a newly expressed transcription factor productively interacts with a target gene in a repressed state in chromatin. In addition, the comparison of the two MMTV templates provides insight into the role of an ordered chromatin structure in the activation process.

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